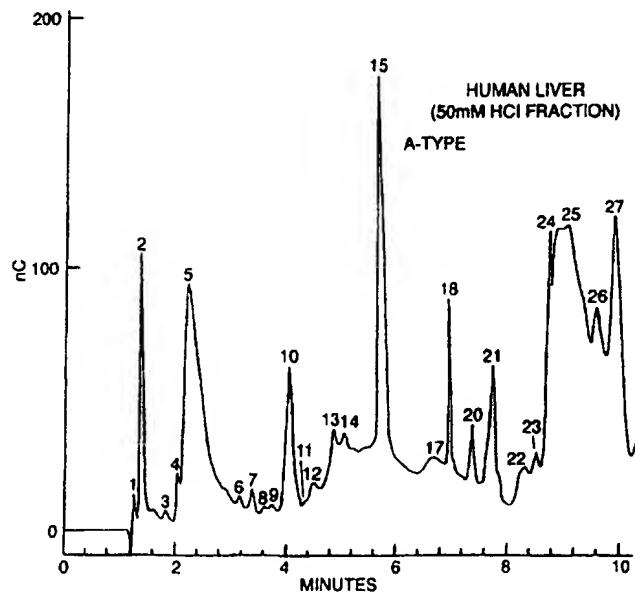




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(54) Title: CYCLITOL CONTAINING CARBOHYDRATES FROM HUMAN TISSUE WHICH REGULATE LIPOGENIC ACTIVITY



(57) Abstract

The application relates to the purification and characterisation of a family of A-type inositolphosphoglycans (IPGs) from human liver and placenta. These substances are shown to have the biological activity associated with A-type IPG fractions, namely regulating lipogenic activity and inhibiting cAMP dependent protein kinase. The characterisation of the compounds demonstrates that they contain metal ions, in particular Zn²⁺, and optionally phosphate. The compounds and their antagonists have uses as pharmaceuticals, e.g. for the treatment of diabetes, and in screening for synthetic analogues.

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Cyclitol Containing Carbohydrates From Human Tissue Which
Regulate Lipogenic Activity

Field of the Invention

5 The present invention relates to the characterisation of second messengers of insulin and other growth factors which regulate lipogenic activity. In particular, the present invention relates to substances which are cyclitol containing carbohydrates, said substances also containing 10 Zn²⁺ ions, to cyclitol containing carbohydrates as obtainable from human liver or human placenta, to compositions comprising these substances and to uses of these substances in methods of medical treatment.

15 Background of the Invention

Many of the actions of growth factors on cells are thought to be mediated by a family of inositol phosphoglycan (IPG) second messengers (TW Rademacher et al, Brazilian J. Med. Biol. Res., 27, 327-341, (1994)). It 20 is thought that the source of IPGs is a "free" form of glycosyl phosphatidylinositol (GPI) situated in cell membranes. IPGs are thought to be released by the action of phosphatidylinositol-specific phospholipases following ligation of growth factor to receptors on the cell surface.

25 There is evidence that IPGs mediate the action of a large number of growth factors including insulin, nerve growth factor, hepatocyte growth factor, insulin-like growth factor I (IGF-I), fibroblast growth factor, transforming growth factor β , the action of IL-2 on B-cells and T-cells, ACTH signalling of adrenocortical cells, IgE, FSH and hCG stimulation of granulosa cells, thyrotropin stimulation of thyroid cells, cell proliferation in the early developing ear and rat mammary gland. However, to date, most of the research in this area has concentrated on 30 the second messengers released by cells in response to insulin. For example, insulin stimulates rapid hydrolysis of membrane-associated GPI molecules in myocytes, adipocytes, hepatoma cells and T-cells. Recently, it has become clear that, at least where insulin is concerned, the 35

released IPGs play an essential role as second messengers, and can in fact mimic many of the effects of insulin in the absence of the hormone.

Soluble IPG fractions have been obtained from a variety of animal tissues including rat tissues (liver, kidney, muscle brain, adipose, heart) and bovine liver. IPG biological activity has also been detected in malaria parasitized RBC and mycobacteria. The ability of an anti-inositolglycan antibody to inhibit insulin action on human placental cytотrophoblasts and BC3H1 myocytes or bovine-derived IPG action on rat diaphragm and chick ganglia suggests cross-species conservation of some three-dimensional features. However, it is well established that species-specific glycoconjugates are a common characteristic and structural characteristics determined on non-human derived IPG may not be found on the human derived material.

We have divided the family of IPG second messengers into distinct A and P-type subfamilies on the basis of their biological activities. In the rat, release of the A and P-type mediators has been shown to be tissue-specific (Kunjara et al, Biopolymers and Bioproducts: Structure, Function and Applications, J. Svast et al (ed), Dokya Publications, 301-306, (1995)). Although in the past it has not been possible to isolate single purified components from the tissue derived IPG fractions, much less in sufficient quantities to allow structural characterisation, there have been studies of the biological activities of the IPG containing fractions, and speculation as to the identity of the active components from non-human sources of the fractions based on indirect evidence from metabolic labelling and cleavage techniques.

Biological activity studies have shown that A-type mediators modulate the activity of a number of insulin-dependent metabolic effects such as acetylCoA carboxylase (activates), cAMP dependent protein kinase (inhibits), adenylate cyclase (inhibits) and cAMP phosphodiesterases

(stimulates). In contrast, P-type mediators modulate the activity of enzymes such as pyruvate dehydrogenase phosphatase (stimulates) and glycogen synthase phosphatase (stimulates). The A-type mediators mimic the lipogenic activity of insulin on adipocytes, whereas the P-type mediators mimic the glycogenic activity of insulin on muscle. Both A and P-type mediators are mitogenic when added to fibroblasts in serum free media. The ability of the mediators to stimulate fibroblast proliferation is enhanced if the cells are transfected with the EGF-receptor. A-type mediators can stimulate cell proliferation in chick cochleovestibular ganglia.

Despite these studies, evidence for the presence of a family of soluble IPG-type mediators in a primary target organ for insulin action in humans has not yet been established. Further, research in this area has been severely hampered by the limited availability of the A and P-type IPGs in fractions derived from mammalian tissues. In particular, there have been experimental difficulties in identifying, isolating and characterising the active components of the IPG fractions having A- and P-type biological activity.

Thus, studies on the measurement in urine of *chiro* and *myo* inositol have been complicated by the fact that both breakdown of endogenous IPGs and dietary sources of the sugars will be present. Accordingly, prior art studies in this area which assumed that the P-type mediator contains *chiro*-inositol and that the A-type mediator contains *myo*-inositol must be interpreted with caution, see Fonteles, MC, Huang, LC, Larner, J, Diabetologia, 39:731-734, (1996), in which the authors report that they incorrectly identified the inositol in the P-type mediator which is pinitol and not *chiro*-inositol. As pinitol is not converted to *chiro*-inositol by the acid conditions used in carbohydrate analysis, this is a case of misidentification.

Further, analysis of material isolated by metabolic labelling with radionuclides or post-isolation labelling of

extracted material cannot be related to the chemical active substance, since one is only following the labelled material and the actual active substance could co-isolate but not be labelled. In addition, various enzymic or 5 chemical treatments of the compounds used to determine structural characteristics inactivate the compound making further structural steps impossible since one can no longer relate activity and structure. Further, as the active components of the A- and P-type IPG fractions are believed 10 to be carbohydrates rather than proteins, they cannot be produced by recombinant DNA technology.

Thus, while there has been speculation in the art as to the chemical identity of these components, to date, there has been no isolation of an active component and no 15 demonstration that it has A- or P-type biological activity.

Summary of the Invention

Our purification reported here from human tissues generates a non-radiolabelled compound which can be 20 visualised on Dionex chromatography and by mass spectrometry. In rats, we can relate changes in the amount of compound present to the insulin stimulation of the tissue. As the rat compounds were isolated by the same protocol as that used to isolate the human compounds, by 25 analogy, the human substances described here are released in response to insulin stimulation. This defines them as insulin-responsive compounds. We have also purified A-type fractions using Vydac HPLC chromatography and shown that the compounds obtained have A-type biological activity.

Broadly, the present invention is based on the isolation of an active component of an A-type IPG fraction derived from human liver in sufficient quantity to characterise this A-type substance for the first time. In particular, this characterisation showed that this 35 substance contains Zn²⁺ ion and has the biological activity associated with A-type IPG fractions, namely regulating lipogenic activity and inhibiting cAMP dependent protein

kinase..

Accordingly, in one aspect, the present invention provides A-type substance which is a cyclitol containing carbohydrate, said substance also containing Zn²⁺ ion and 5 optionally phosphate and having the properties of regulating lipogenic activity. This finding was made as some of the A-type fractions isolated using Vydac HPLC chromatography did not contain phosphate but were biologically active, indicating that phosphate is not 10 essential for biological activity.

Accordingly, in the present application, references to "inositolphosphoglycans" or "IPGs" include compounds in which phosphate is not present. These compounds are alternatively be termed inositolglycans (IGs).

15 We have further found the A-type substance to have the following properties:

1. Migrates near the origin in descending paper chromatography using 4/1/1 butanol/ethanol/water as a solvent.
- 20 2. Some of the substances contains phosphate which is directly related to activity.
3. They are bound on Dowex AG50 (H⁺) cation exchange resin.
4. Does not bind to a C18 affinity column.
- 25 5. They are bound on an AG3A anion exchange resin.
6. The activity is resistant to pronase.
7. They are detected using a Dionex chromatography system or Vydac HPLC chromatography (see figures 7 to 9).

30 The substance may also have one or more of the following activities associated with A-type IPG fractions:

- (a) inhibits adenylate cyclase;
- (b) mitogenic when added to EGF-transfected fibroblasts in serum free medium.
- (c) stimulates lipogenesis in adipocytes

35 Thus, while the prior art discloses that the biological activities associated with A-type IPG can be detected in fractions obtained from bovine and rat tissues,

it does not isolate or characterise the component from the fraction and demonstrate that it has a A-type IPG biological activity.

5 In a further aspect, the present invention provides a substance which is a cyclitol containing carbohydrate, said substance also containing Zn^{2+} ion and optionally phosphate, as obtainable by from human liver or placenta by:

10 (a) making an extract by heat and acid treatment of a liver homogenate, the homogenate being processed from tissue immediately frozen in liquid nitrogen;

(b) after centrifugation and charcoal treatment, allowing the resulting solution to interact overnight with an AG1-X8 (formate form) anion exchange resin;

15 (c) collecting a fraction having A-type IPG activity obtained by eluting the column with 50 mM HCl;

(d) neutralising to pH4 (pH not to exceed 7.8) and lyophilising the fraction to isolate the substance;

(e) descending paper chromatography using 4/1/1 butanol/ethanol/water as solvent;

20 (f) purification using high-voltage paper electrophoresis in pyridine/acetic acid/water; and,

(g) purification using Dionex anion exchange chromatography, or purification and isolation using Vydac HPLC chromatography.

25 In a further aspect, the present invention provides an isolated substance which is an A-type cyclitol containing carbohydrate comprising Zn^{2+} ion and has the biological activity of regulating lipogenic activity and inhibiting cAMP dependent protein kinase, wherein the substance has:

30 (a) a molecular weight determined using negative mode MALDI mass spectroscopy as shown in tables 3 and 4, or a molecular weight related to one of the molecular weights set out in tables 3 and 4 by the addition or subtraction of one or more 211m/z structure units; or,

35 (b) a molecular weight determined using positive mode MALDI mass spectroscopy as shown in table 5, or a molecular weight related to one of the molecular weights set out in

table 5 by the addition or subtraction of one or more 211m/z structure units.

In a further aspect, the present invention provides pharmaceutical compositions comprising a substance as described above, optionally in combination with insulin or P-type substances for simultaneous or sequential administration. These compositions can be used in the treatment of disorders in which the lipogenic response of a patient has in some way been affected so that they produce a relatively low amount of A-type IPGs in response to growth factors such as insulin.

In a further aspect, the present invention provides antagonists to the substances described above and pharmaceutical compositions comprising these antagonists. These compositions can be useful in the treatment of conditions in which A-type IPGs are overproduced, e.g. in obese NIDDM patients, and/or to antagonise one of the activities of the A-type IPGs. Such an antagonist may be a related IPG which is able to compete with the A-type IPG but have no biological action in its own right.

In a further aspect, the present invention provides a substance or antagonist as described above for use in a method of medical treatment.

It is expected that synthetic compounds containing all or part of the active substituents of the A-type IPG could be useful as therapeutics.

Brief Description of the Drawings

Figure 1 shows DX500 HPLC of purified A-type family of mediators from human liver.

Figure 2 shows the stimulation of EGFR T17 fibroblasts and PDH phosphatase. Serial dilutions of stock human liver derived type-A and type-P were assayed for their ability to stimulate proliferation. Control represents the proliferation of the fibroblasts in serum-free medium with addition of IPG.

Figure 3 shows the purification of IPG by descending

paper chromatography. Descending paper chromatography profiles of control- and pronase E-treated IPG type-A and type-P, panels A and B respectively, following analysis for phosphate content. Panels C and D show the free amino groups analysis in the same chromatographic fractions. For clarity, only the first 10 fractions are displayed in each panel for pronase treated samples. The profiles for untreated mediators was identical. The solvent front was +35cm.

Figure 4 shows the high voltage electrophoresis of IPG type-A and type-P mediators. Figure 4a shows a representative electrophoretogram of IPG type-P (black) HVE following detection of phosphate. The migration positions of bromophenol blue (BB), inositol monophosphate (IP1) and inositol di/tri-phosphate are indicated by arrows.

Figure 5 shows the quantitative increase in IPG release following infusion with insulin.

Figure 6 the family of IPGs responsive to insulin as detected by DX500 anion exchange chromatography. Peaks with * are not present in the pre-insulin stimulated rat liver.

Figure 7 shows the phosphate content of a family of A-type substances isolated and purified using Vydac HPLC chromatography.

Figure 8 shows the bioactivity of selected A-type substances isolated and purified using Vydac HPLC chromatography.

Figure 9 shows the Dionex peak of the A-type fraction isolated using Vydac HPLC chromatography, showing that this fraction corresponds to peak 23 in figures 1 and 6.

Figures 10 and 11 show high resolution MALDI mass spectra (negative mode) of the family of A-type IPGs isolated from liver after insulin stimulation. The main peaks in the spectra are set out in tables 3 and 4.

Figure 12 shows the corresponding high resolution MALDI mass spectrum (positive mode) of the family of A-type IPGs. The main peaks in the spectra are set out in table

5.

Detailed DescriptionMimetic Design

5 The designing of mimetics to a known pharmaceutically active compound is a known approach to the development of pharmaceuticals based on a "lead" compound. This might be desirable where the active compound is difficult or expensive to synthesise or where it is unsuitable for a particular method of administration, e.g. peptides are 10 unsuitable active agents for oral compositions as they tend to be quickly degraded by proteases in the alimentary canal. Mimetic design, synthesis and testing is generally used to avoid randomly screening large number of molecules 15 for a target property.

There are several steps commonly taken in the design of a mimetic from a compound having a given target property. Firstly, the particular parts of the compound that are critical and/or important in determining the 20 target property are determined. These parts of the compound constituting its active region are known as its "pharmacophore".

Once the pharmacophore has been found, its structure is modelled to according its physical properties, eg 25 stereochemistry, bonding, size and/or charge, using data from a range of sources, e.g. spectroscopic techniques, X-ray diffraction data and NMR. Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modelling process.

In a variant of this approach, the three-dimensional structure of the ligand and its binding partner are modelled. This can be especially useful where the ligand and/or binding partner change conformation on binding, 30 allowing the model to take account of this the design of the mimetic.

A template molecule is then selected onto which

chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted on to it can conveniently be selected so that the mimetic is easy to synthesise, is likely to be 5 pharmacologically acceptable, and does not degrade *in vivo*, while retaining the biological activity of the lead compound. The mimetic or mimetics found by this approach can then be screened to see whether they have the target 10 property, or to what extent they exhibit it. Further optimisation or modification can then be carried out to arrive at one or more final mimetics for *in vivo* or clinical testing.

In the present case, it is expected that synthetic compounds containing all or part of the active substituents 15 of the A-type IPG could be useful as therapeutics.

Antagonists

Antagonists to the A-type substances include substances which have one or more of the following 20 properties:

(a) substances capable of inhibiting release of the A-type mediators;

(b) substances capable of reducing the levels of A-type mediators via a binding substance (e.g. an antibody or 25 specific binding protein); and/or,

(c) substances capable of reducing the effects of A-type mediators.

In one embodiment, the IPG antagonists are specific binding proteins. Naturally occurring specific binding 30 proteins can be obtained by screening biological samples for proteins that bind to IPGs.

In a further embodiment, the antagonists are antibodies capable of specifically binding to A-type IPGs. The production of polyclonal and monoclonal antibodies is 35 well established in the art. Monoclonal antibodies can be subjected to the techniques of recombinant DNA technology to produce other antibodies or chimeric molecules which

retain the specificity of the original antibody. Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the complementarity determining regions (CDRs), of an antibody to the constant regions, or constant regions plus framework regions, of a different immunoglobulin. See, for instance, EP-A-184187, GB-A-2188638 or EP-A-239400. A hybridoma producing a monoclonal antibody may be subject to genetic mutation or other changes, which may or may not alter the binding specificity of antibodies produced.

Antibodies may be obtained using techniques which are standard in the art. Methods of producing antibodies include immunising a mammal (e.g. mouse, rat, rabbit, horse, goat, sheep or monkey) with the protein or a fragment thereof. Antibodies may be obtained from immunised animals using any of a variety of techniques known in the art, and screened, preferably using binding of antibody to antigen of interest. For instance, Western blotting techniques or immunoprecipitation may be used (Armitage et al, Nature, 357:80-82, 1992). Isolation of antibodies and/or antibody-producing cells from an animal may be accompanied by a step of sacrificing the animal.

As an alternative or supplement to immunising a mammal with a peptide, an antibody specific for a protein may be obtained from a recombinantly produced library of expressed immunoglobulin variable domains, e.g. using lambda bacteriophage or filamentous bacteriophage which display functional immunoglobulin binding domains on their surfaces; for instance see WO92/01047. The library may be naive, that is constructed from sequences obtained from an organism which has not been immunised with any of the proteins (or fragments), or may be one constructed using sequences obtained from an organism which has been exposed to the antigen of interest.

Antibodies according to the present invention may be modified in a number of ways. Indeed the term "antibody" should be construed as covering any binding substance

having a binding domain with the required specificity. Thus the invention covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including synthetic molecules and molecules whose shape mimics that of an antibody enabling it to bind an antigen or epitope.

Example antibody fragments, capable of binding an antigen or other binding partner are the Fab fragment consisting of the VL, VH, Cl and CH1 domains; the Fd fragment consisting of the VH and CH1 domains; the Fv fragment consisting of the VL and VH domains of a single arm of an antibody; the dAb fragment which consists of a VH domain; isolated CDR regions and F(ab')₂ fragments, a bivalent fragment including two Fab fragments linked by a disulphide bridge at the hinge region. Single chain Fv fragments are also included.

Humanised antibodies in which CDRs from a non-human source are grafted onto human framework regions, typically with the alteration of some of the framework amino acid residues, to provide antibodies which are less immunogenic than the parent non-human antibodies, are also included within the present invention

A hybridoma producing a monoclonal antibody according to the present invention may be subject to genetic mutation or other changes. It will further be understood by those skilled in the art that a monoclonal antibody can be subjected to the techniques of recombinant DNA technology to produce other antibodies or chimeric molecules which retain the specificity of the original antibody. Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the complementarity determining regions (CDRs), of an antibody to the constant regions, or constant regions plus framework regions, of a different immunoglobulin. See, for instance, EP-A-184187, GB-A-2188638 or EP-A-0239400. Cloning and expression of chimeric antibodies are described in EP-A-0120694 and EP-A-0125023.

Hybridomas capable of producing antibody with desired binding characteristics are within the scope of the present invention, as are host cells, eukaryotic or prokaryotic, containing nucleic acid encoding antibodies (including antibody fragments) and capable of their expression. The invention also provides methods of production of the antibodies including growing a cell capable of producing the antibody under conditions in which the antibody is produced, and preferably secreted.

The antibodies described above may also be employed in the diagnostic aspects of the invention by tagging them with a label or reporter molecule which can directly or indirectly generate detectable, and preferably measurable, signals. The linkage of reporter molecules may be directly or indirectly, covalently, e.g. via a peptide bond or non-covalently. Linkage via a peptide bond may be as a result of recombinant expression of a gene fusion encoding antibody and reporter molecule.

One favoured mode is by covalent linkage of each antibody with an individual fluorochrome, phosphor or laser dye with spectrally isolated absorption or emission characteristics. Suitable fluorochromes include fluorescein, rhodamine, phycoerythrin and Texas Red. Suitable chromogenic dyes include diaminobenzidine.

Other reporters include macromolecular colloidal particles or particulate material such as latex beads that are coloured, magnetic or paramagnetic, and biologically or chemically active agents that can directly or indirectly cause detectable signals to be visually observed, electronically detected or otherwise recorded. These molecules may be enzymes which catalyse reactions that develop or change colours or cause changes in electrical properties, for example. They may be molecularly excitable, such that electronic transitions between energy states result in characteristic spectral absorptions or emissions. They may include chemical entities used in conjunction with biosensors. Biotin/avidin or

biotin/streptavidin and alkaline phosphatase detection systems may be employed.

In a further embodiment, the IPG antagonists are synthetic compounds. These may be produced by conventional chemical techniques or using combinatorial chemistry, and then screened for IPG antagonist activity. These compounds may be useful in themselves or may be used in the design of mimetics, providing candidate lead compounds for development as pharmaceuticals. Synthetic compounds might be desirable where they are comparatively easy to synthesize or where they have properties that make them suitable for administration as pharmaceuticals, e.g. antagonist which are peptides may be unsuitable active agents for oral compositions if they are degraded by proteases in the alimentary canal. Mimetic design, synthesis and testing is generally used to avoid randomly screening large number of molecules for a target property.

Pharmaceutical compositions

The mediators and antagonists of the invention can be formulated in pharmaceutical compositions. These compositions may comprise, in addition to one or more of the mediators or antagonists, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of administration, e.g. oral, intravenous, cutaneous or subcutaneous, nasal, intramuscular, intraperitoneal routes.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide

solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

Whether it is a polypeptide, antibody, peptide, small molecule or other pharmaceutically useful compound according to the present invention that is to be given to an individual, administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of the techniques and protocols mentioned above can be found in Remington's Pharmaceutical Sciences, 16th edition, Osol, A. (ed), 1980. In a preferred embodiment, dosage levels will be determined as producing euglycaemic conditions.

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

Diagnostic Methods

Methods for determining the concentration of analytes in biological samples from individuals are well known in the art and can be employed in the context of the present invention to determine the ratio of P- and A-type inositolphosphoglycans (IPGs) in a biological sample from a patient. This in turn can allow a physician to determine if the ratio or level of P- and A-type IPGs is out of balance having regard to the patient and the condition being tested for. Examples of diagnostic methods are described in the experimental section below.

Preferred diagnostic methods rely on the determination of the ratio of P- and A-type IPGs. The methods can employ biological samples such as blood, serum, tissue samples or urine.

The assay methods for determining the concentration of P- and A-type IPGs typically employ binding agents having binding sites capable of specifically binding to one or more of the P- or A-type IPGs in preference to other molecules. Examples of binding agents include antibodies, receptors and other molecules capable of specifically binding the IPGs. Conveniently, the binding agent(s) are immobilised on solid support, e.g. at defined locations, to make them easy to manipulate during the assay.

The sample is generally contacted with the binding agent(s) under appropriate conditions so that P-and A-type IPGs present in the sample can bind to the binding agent(s). The fractional occupancy of the binding sites of the binding agent(s) can then be determined using a developing agent or agents. Typically, the developing agents are labelled (e.g. with radioactive, fluorescent or enzyme labels) so that they can be detected using techniques well known in the art. Thus, radioactive labels can be detected using a scintillation counter or other radiation counting device, fluorescent labels using a laser and confocal microscope, and enzyme labels by the action of an enzyme label on a substrate, typically to produce a colour change. The developing agent(s) can be used in a

competitive method in which the developing agent competes with the analyte for occupied binding sites of the binding agent, or non-competitive method, in which the labelled developing agent binds analyte bound by the binding agent or to occupied binding sites. Both methods provide an indication of the number of the binding sites occupied by the analyte, and hence the concentration of the analyte in the sample, e.g. by comparison with standards obtained using samples containing known concentrations of the analyte. In preferred embodiments, this can then be used to determine the P:A type ratio.

Methods

Isolation and characterisation of inositol phosphoglycans.

Inositolphosphoglycans (IPG) were purified as follows. from human liver. The frozen tissue (90g) was powdered under liquid nitrogen and placed directly into boiling 50 mM formic acid containing 1 mM EDTA and 1 mM 2-mercaptoethanol (3 mL of buffer per gram (wet weight) of tissue). After 1 min homogenisation with a polytron mixer (Kinematica, Littau, Switzerland), the solution was further boiled for 5 min. The solution was then cooled on ice and centrifuged at 29,500g for 2 h at 4°C. The supernatant was treated with 10 mg/mL activated charcoal for 30 min with stirring at 4°C. The charcoal suspension was centrifuged at 29,500g for 1 h at 4°C and the clear supernatant recovered. The solution was then diluted ten-fold with distilled water, adjusted to pH 6.0 with 10% NH₄OH solution and then gently shaken overnight at room temperature with AG1-X8 (formate form) resin (0.3 mL resin per mL solution). The resin was then poured into a chromatography column (2.5 x 60 cm) and washed sequentially with water (2 bed volumes) and 1 mM HCl (2 bed volumes). Then, the material was eluted with 50 mM HCl (5 bed volumes) to obtain an IPG A-type fraction. This fraction was adjusted to pH 4.0 with 10% NH₄OH solution and then dried in a rotary evaporator. The dried material was redissolved in distilled water,

lyophilised twice and divided into five aliquots for both chemical and biochemical analyses. For analyses, aliquots of the A-type preparation were dissolved in 200 µL of Hanks Medium and adjusted to pH 7.0 with 1 M KOH. For human tissue the mediators extracted from the equivalent of 16 g (wet weight) of tissue were dissolved in a final volume of 200 µL (stock solution). Therefore, 10 µL of stock represents the amount of A-type mediator recovered from 800 mg of starting tissue.

10

Pronase treatment.

IPG were treated with Pronase E as described elsewhere. Briefly, a stock solution of the enzyme (10 mg/mL) was preincubated at 60°C for 30 min in 100 mM Tris-HCl buffer, pH 7.8, to inactivate contaminating enzymes which may be present. Digestion of the sample was started by addition of pronase solution (30 µL) to IPG samples in 200 µL of 100 mM Tris-HCl buffer at pH 8.0 at 37°C. After two hours, the reaction was terminated by boiling for 3 min and removed by acid precipitation.

IPG purification by paper chromatography.

IPG were dissolved in a minimum amount of water and applied to a 3MM chromatography paper (3 x 50 cm, origin at 8.5 cm). Descending paper chromatography was performed using n-butanol/ethanol/water (4:1:1, v/v/v) and the chromatogram was developed for 9 h. After drying, the paper was cut every centimeter (-1 to +35 cm from the origin) and the material associated to each fraction eluted with water (60 µL, 5 washes). Each fraction was evaporated to dryness and redissolved either in water or in Hanks solution (60 µL) and neutralized with 1 N KOH prior to the determination of free amino groups, phosphate content or to assay biological activities.

35

High voltage paper electrophoresis.

The material eluted from fractions 1 to 6 after paper

chromatography, was pooled, redissolved in a small volume of water and applied to a 3MM electrophoresis paper. Bromophenol blue and tritiated inositol phosphates mixture were added as standards. The samples were electrophoresed 5 for 30 min at 80 Vcm⁻¹ in pyridine/acetic acid/water (3:1:387, v/v/v), pH 5.4. Neutral compounds remained at the point of application, while negatively charged compounds moved towards the anode. After the paper was dried, fractions were cut out every one cm and eluted with 10 water.

Vydac HPLC chromatography

This technique was used to isolate and purify individual fractions containing the mediators. The A-type 15 IPG was applied to a Vydac 301 PLX575 HPLC column. The column was eluted as follows:

Solvent: Ammonium acetate 500mM pH 5.5,
Gradient conditions: 0-5% over 12 minutes,
5-21% over the next 13 minutes,
20 21-80% over 25 minutes,
80-100% over 5 minutes.

The fractions were then assayed for phosphate and growth promoting activity using EGF-transfected fibroblasts.

25

Determination of free amino groups.

Measurement of free amino groups was performed as described below. Samples and standards (0-100 nmol of D-(+)-glucosamine hydrochloride, Sigma) were dissolved in ultrapure water (50 µL) before sequentially adding sodium borate (0.14 M, pH 9) and fluorescamine (0.75 mg/ml prepared in dry acetone). Emission fluorescence at 475 nm was observed after excitation at 390 nm using a spectrofluorimeter.

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Determination of phosphate content.

Total phosphate levels were assayed as described

below. Samples and standards (0-100 nmoles of Na₂HPO₄) were evaporated to dryness and hydrolysed with perchloric acid (70%) at 180°C for 30 min. After cooling to room temperature, ultrapure water (250 µL), (NH₄)₂MoO₄ (100 µL of a 2.5% solution) and ascorbic acid (100 µL of a 10% solution) were sequentially added. Colour development was achieved by heating the samples at 95 °C for 15 min. Optical absorbance was measured at 655 nm in a microplate reader.

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Interaction of IPG with Ion Exchange resins and Sep-Pak C18 cartridges.

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Thirty microliters of stock solution (see above) were loaded onto columns containing 600 µL of either AG3-X4 (HO⁻), AG50-X12 (H⁺) or onto Sep Pack C18 cartridges and then eluted with water (5 bed volumes). The solutions were concentrated to dryness and the residues obtained re-dissolved in 30 µL of Hanks and adjusted to pH 7.0.

20

Evaluation of cAMP-dependent protein kinase activity.

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The ability of the IPG fraction to inhibit the activity of the cyclic AMP-dependent protein kinase was assessed by using histone IIA as substrate. The reaction mixture (100 µL) contained 25 mM HEPES buffer (pH 7.6), 10 µM MgATP (10⁶ cpm [γ -³²P]ATP), histone IIA (50 µg protein), and catalytic subunit of PKA (60 units/mL). In all the determinations, 10 µL of IPG solution (see above) was added to the reaction mixture. After incubation at 37°C for 10 min, the reaction was stopped and proteins precipitated with 10% trichloroacetic acid (100 µL) and 2% bovine serum albumin (10 µL) and the incorporation of ³²P into proteins was determined.

30

Evaluation of the pyruvate dehydrogenase phosphatase (PDH) activity.

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Pyruvate dehydrogenase complex (PDC) and the PDH phosphatase were prepared and stored at -80°C until use.

The assay for both PDH phosphatases, in the presence or absence of insulin mediator, was based upon the initial rate of activation of inactivated, phosphorylated PDH complex. The initial activity of the PDC was 8-13 units/ml (1 unit of enzyme produces 1 μ mol NADH/min) and after inactivation with ATP, 0.3-0.5 units/ml (inactivated PDC). A two stage assay was used to quantitate the phosphatase activity. A sample of inactivated PDC (50 μ L) was preincubated at 30°C with 1 mg/mL fat-free BSA, 10 mM MgCl₂, 10 mM CaCl₂, and 1 mM DTT in 20 mM potassium phosphate buffer at pH 7.0 (total volume 250 μ L) for three minutes. At this time, 10 μ L of the PDH phosphatase and 10 μ L of IPG were added and the incubation continued for a further 2 min. At the end of this time, 200 μ L of the mix was removed and added to 100 μ L of 300 mM NaF. The activated PDH was determined at the second stage photometrically by measuring the rate of production of NADH. One hundred microliters of the stopped reaction were added to 1 mL of reaction mixture containing 50 mM potassium phosphate buffer at pH 8.0, 2.5 mM β -NAD⁺, 0.2 mM TPP, 0.13 mM coenzyme A, 0.32 mM DTT and 2 mM sodium pyruvate. The production of NADH was followed at 340 nm for 5 min.

Activation of lipogenesis in rat adipocytes.

This was carried out on adipocytes isolated from the epididymal fat pads of young rats. Briefly adipocytes from two rat epididymial fat pads were suspended in 8ml of Krebs Ringer bicarbonate and 250 μ L of the cell suspension was incubated at 37°C for two hours in Krebs Ringer bicarbonate, containing 1% albumin and 5 mM [U-¹⁴C]-glucose (1 μ Ci/sample) with or without insulin (1 nM) or 10 μ L of IPG solutions. The rate of incorporation of uniformly labelled glucose into fatty acids was used as a measure of lipogenesis.

35

Measurement of cellular proliferation in fibroblasts.

EGFR17 fibroblasts were routinely grown in

	A1			
#1	8.83	0.91	19.84	8.72 μ g/ml Zn
#2	8.75	1.00	19.26	0.96 μ g/ml Mn
#3	8.58	0.97	19.58	19.56 μ g/ml Fe
5				
	A2			
#1	7.74	0.91	18.88	7.66 μ g/ml Zn
#2	7.64	0.86	18.40	0.88 μ g/ml Mn
#3	7.61	0.88	19.12	18.80 μ g/ml Fe
10				
	Blank			
#1	-	-	10.24	
#2	-	-	10.26	10.28 μ g/ml Fe
#3	-	-	10.33	
15				
	Blank			
#1	-	-	8.42	8.32 μ g/ml Fe
#2	-	-	8.21	
20	Average of Blank 1 and 2 = 9.3 μ g/ml Fe			

This shows for the first time that the A-type IPG isolated from human liver contains Zn²⁺ ion.

25 Inhibition of cAMP dependent protein kinase.

The ability of the IPG to inhibit cAMP-dependent protein kinase was tested by determining the incorporation of ³²P into washed histone IIA. The addition of the A-type IPG fraction diluted 1/10, caused 78.5±9.5 (n=4) percent inhibition of the kinase activity. This effect was dose dependent and in a typical experiment, a significant inhibition of the kinase activity was achieved with the A-type fraction at a final concentration of 1/100 or less (50% inhibition A-type). This experiment was in agreement with our previous data for rat-derived IPGs which showed that the A-type fraction contains the predominant inhibitor activity against cAMP dependent protein kinase. We have

Dulbecco's Modified Eagle's Medium (DMEM) containing 10% v/v foetal calf serum, 2 mM L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO₂. The cells were subcultured when they approached 80 % confluence. The EGFRT17 cells are NIH 3T3 fibroblasts transfected with the human epidermal growth factor receptor [32,35]. To evaluate fibroblast cell proliferation, cells were plated into 96-well microtitre wells at a density of 10⁴ cell per well in DMEM containing 10% FCS. After 24 h the medium was removed, the cells washed twice with Hanks medium, serum free medium was added, and the cells were incubated for a further 24 h period. At this point the cells were stimulated with serum, IPG preparations or the appropriate controls. Eighteen hours later [³H]thymidine (1 µCi/well), the was added to each well for 4 h. At the end of this treatment, the cells were washed twice with Hanks solution, trypsinised, and radioactivity associated with cellular DNA determined using a cell harvester. For the cell proliferation assays, the dilutions are final dilutions. For example 2.5 µL of the stock solution is added to a final volume of 100 µL, or 1/40 dilution.

Protocol for sandwich ELISA.

The protocol below sets out an indirect, non-competitive, solid-phase enzyme immunoassay (sandwich ELISA) for the quantification of inositolphosphoglycans (IPG) in biological fluids, such as human serum.

In the assay, monoclonal IgM antibodies are immobilised on a solid phase. Tissue culture supernatant, ascitic fluid from mice with a peritoneal tumour induced by injecting hybridoma cells into the peritoneum and purified monoclonal antibody have been used in the immunoassay. F96 Maxisorp Nunc-Immuno plates were used for these assays. Maxisorp surface is recommended where proteins, specially glycoproteins such as antibodies, are bound to the plastic.

The immobilised antibody captures the antigen from the

test sample (human serum or IPG used like a positive control).

5 A bridging antibody (a purified polyclonal IPG antibody from rabbit) is needed to link the anti-antibody biotinylated to the antigen.

10 The detection method employs an anti-rabbit Ig, biotinylated species-specific whole antibody (from donkey) and a streptavidin-biotinylated horseradish peroxidase complex (Amersham), ABTS and buffer for ABTS (Boehringer Mannheim).

The ELISA assay can be carried out as follows:

1. Add 100 µl/well in all the steps.
2. Add monoclonal antibody diluted 1:100 in PBS in a F96 Maxisorp Nunc-Immuno plate. Incubate at least 2 days at
15 4°C.
3. Wash with PBS three times.
4. Add a blocking reagent for ELISA (Boehringer Mannheim) in distilled water (1:9) 2 hours at room temperature.
5. Wash with PBS-Tween 20 (0,1%) three times.
6. Add a purified polyclonal antibody (diluted 1:100 in PBS), overnight at 4°C.
7. Wash with PBS-Tween 20 (0.1%) three times.
8. Add an anti-rabbit Ig, biotinylated species-specific whole antibody (from donkey) (Amersham) diluted 1:1000 in PBS, 1 h 30 min at room temperature.
9. Wash with PBS-Tween 20 (0.1%) three times.
10. Add a streptavidin-biotinylated horseradish peroxidase complex (Amersham) diluted 1:500 in PBS, 1 h 30 min at room temperature.
11. Wash with PBS three times.
12. Add 2,2-Azino-di-(3-ethylbenzthiazoline sulfonate (6)) diammonium salt crystals (ABTS) (Boehringer Mannheim) to buffer for ABTS (BM): Buffer for ABTS is added to distilled water (1:9 v/v). 1mg of ABTS is added to 1 ml of diluted buffer for ABTS.
13. Read the absorbance in a Multiscan Plus P 2.01 using a 405 nm filter in 5-15 min.

Results

IPG isolation and biological activities.

IPGs were extracted from human liver and placenta as follows. Briefly, the extract was prepared by heat and acid treatment of a liver homogenate, processed from tissue immediately frozen in liquid nitrogen after removal from the patient, therefore preventing the action of phosphatases. After centrifugation and charcoal treatment, the solution was allowed to interact overnight with an anion exchange resin (AG1-X8, formate form). The resin was washed sequentially with water and dilute hydrochloric acid. Elution with 50 mM HCl produced an A-type fraction which stimulated lipogenesis in adipocytes and stimulated proliferation of EGF receptor transfected fibroblasts. This fraction was then subjected to descending paper chromatography using 4/1/1 butanol/ethanol/water as solvent, followed by purification using high-voltage paper electrophoresis in pyridine/acetic acid/water, and finally purification using Dionex (trade mark) anion exchange chromatography. Fig 1 shows a sharp spike (fraction no. 15) representing the major A-type substance. This fraction was then neutralized and lyophilised several times.

Metal ion analysis.

Metal ion analysis was performed on a DX500 system with visible detection at 520nm.

The separation was achieved using IonPac mixed bed ion exchange columns with pyridine-2,6-dicarboxylic acid eluent and post column reaction with pyridial azo resorcinol. The A-type samples (A1, A2 and A3) were reconstituted in 100 μ l of water. 10 μ l of this solution was taken, 10 μ l of conc HCl added and the sample left overnight. Then, 80 μ l of water was added to the mixture and 10 μ l of this solution was analysed. Blank HCl samples acted as controls.

μ g/ml Zn	μ g/ml Mn	μ g/ml Fe	Averages
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previously used the nomenclature A-type to denote the IPG family of compounds present in the 50 mM fraction, to highlight this activity.

5 **Stimulation of PDH phosphatase.**

The ability of the A-type fraction to stimulate pyruvate dehydrogenase phosphatase isolated from bovine heart was determined. Both human livers contained a limited level of PDH phosphatase stimulating activity in 10 the A-type eluate, in line with results from the prior art rat studies, containing on average 2.8 fold less activity compared to rat P-type IPG fractions.

Effect on lipogenesis.

15 The fraction containing the putative A-type IPG was added to rat adipocytes and the ability of this fraction to stimulate lipogenesis determined. Table 1 shows that the A-type fraction contained lipogenic activity. Human liver was found to contain the same amount of lipogenic activity 20 as the insulin stimulated rat liver.

Effects on NIH 3T3 fibroblast proliferation.

The A-type IPG fraction was assayed for its ability to support proliferation of fibroblasts in the absence of serum. For rat tissue this assay has been used to estimate 25 the relative abundance of the two mediators since both A-type mediators are active in the assay [10,26,27]. The A-type human liver derived-fraction was found to be mitogenic when added to fibroblasts transfected with the EGF receptor 30 in serum-free media. Figure 2 shows the dose-dependent effect obtained for the fraction. Saturation was not yet obtained at the highest concentrations used. The fraction was however able to induce proliferation at least 2-2.5 fold greater than 10% FCS alone.

35

Descending paper chromatography.

A portion of the A-type material obtained was treated

with Pronase E for 2 hours and then the pronase removed by acid precipitation. The solution remaining was concentrated, redissolved in water and subjected to purification by descending paper chromatography using n-butanol/ethanol/ water. After development for 9 hours, the presence of phosphate and free amino groups was detected. Figure 3a shows the chromatogram profiles for the putative A-type mediator, following analysis for phosphate. Compounds containing phosphate were found to migrate between the origin and ~5cm. The paper chromatograms were also analysed for the presence of free amine groups as shown in Figure 3c and d. Again compounds containing free amino groups were present between the origin and a migration distance of 5 cm. Incubation with pronase made no difference to the migration of the compounds as assessed by the phosphate analysis as shown in Figure 3a.

Interaction with Ion Exchange resins and Sep-Pak C18 cartridges.

The behaviour of IPG in its interaction with two different ion exchange resins and a reverse phase C18 column were determined by the ability of the eluates to induce thymidine incorporation into EGFR17 DNA cells. The results are shown in Table 2. In the case of the reverse phase, about 80-85% of the biological activity was recovered with water elution for the A-type IPG. These results demonstrate that the A-type mediator is hydrophilic. Table 2 also shows that the A-type mediators could not be recovered from either a cation exchange column (AG50-X12) or an anion exchange column (AG3-X4). This is consistent with the presence of dual functional groups such as free amino and phosphate moieties.

Activity requires metal ions.

The IPGs were extracted with dithizone (see Appendix 1, section 2) to remove all metal ions. Following extraction, the A-type IPG was inactive in the lipogenic

assay.

IPG containing carbohydrates.

Chromatograms of purified IPG A-type from human liver
5 were detected by pulse-amperimetic detections (conditions given in Appendix 1, section 4).

The presence of various forms of inositol (myo-inositol, chiro-inositol, pinitol) was confirmed using a DX500 system and a Carbo Pac MA1 column and pulsed amperimetic detection (method given in Appendix 1, section 10 6).

Purification by high-voltage electrophoresis (HVE).

The material eluted from the paper after descending chromatography was subjected to high-voltage paper electrophoresis at pH 5.4. Under these conditions, negatively charged compounds containing phosphate, carboxy, or sulphate groups migrate towards the anode. A representative paper electrophoretogram of three independent experiments is shown in Figure 4a following analysis for phosphate. Phosphate was detected at the origin and as a broad unresolved peak extending from 5 cm to 20 cm migration distance. The presence of phosphate at the origin indicates that compounds recovered in this position must have an equal number of positively charged moieties which neutralize the overall charge. Those compounds which migrate either have an excess of negatively charged groups (e.g. phosphate) over positively charged moieties (e.g. amino, metal). The activity profile of the 20 A-type mediators following HVE very closely mirrors the phosphate analysis shown in Figure 4a with activity present 25 at the origin and in a broad band extending to 20 cm 30 migration distance.

35 Vydac HPLC Chromatography

In order to demonstrate that it is possible to isolate and purify A-type mediators from samples containing the

family of compounds shown in figure 1, fractions obtained from a Vydac 301 PLX575 HPLC column and were analysed for phosphate and growth promoting activity. Figure 7 shows the phosphate levels of the different fractions and figure 5 8 shows the bioactivity of the selected fractions including 5, 14, 25, 28, 35 and 39. The predominant growth promoting activity was found in fractions 25-39. The Dionex HPLC profile of the main active fraction is shown in figure 9. This fraction contains predominantly peak 23 shown in 10 figures 1 and 6.

MALDI mass spectroscopy

Figures 10 and 11 show high resolution MALDI mass spectra (negative mode) of the family of A-type molecules 15 isolated from liver, while figure 12 shows the corresponding positive MALDI mass spectrum for the same sample. The family of structures are related by the addition of 211m/z structure units, e.g. 808m/z \Rightarrow 1019m/z \Rightarrow 1239m/z \Rightarrow 1441m/z. Thus, from each low molecular weight 20 peak, this relationship can be used to find the molecular weights of other A-type mediators in the family. The molecular weights of the A-type substances are shown in tables 3 to 5.

The molecular weights determined by negative mode 25 MALDI mass spectroscopy differ from the actual molecular weights of the A-type substances by the removal of a H' atom, i.e. the actual weight can be obtained by adding +1 to the weights set out in tables 3 and 4. The positive mode increases in m/z as compared to the negative mode by 30 59 which indicates the addition of one Na⁺ and K⁺ atom to each component with the concomitant loss of two protons (i.e 39 + 22 - 2 = 59). Thus, the molecular weight of the P-type substances can be determined by subtracting 57 from 35 the molecular weights set out in table 5. Thus, it is straightforward to determine the molecular weights based on the results in the tables.

Monoclonal antibodies.

Inositolphosphoglycan (IPG) purified from rat liver by sequential thin layer chromatography (TLC) was used to immunise New Zealand rabbits and Balb/c mice by using conventional procedures.

After immunisation, monoclonal antibodies were prepared using the approach of fusion of mouse splenocytes (5×10^6 cells/ml) with mutant myeloma cells (10^6 cells/ml). The myeloma cell lines used were those lacking hypoxanthine-guanine phosphoribasyl transferase. The screening method of hybridoma cells was based on a non-competitive solid-phase enzyme immunoassay in which the antigen (IPG) was immobilised on a solid phase. Culture supernatants were added and positive hybridoma cells were selected.

A single cell cloning was made by limiting dilution. Hybridomas for three monoclonal antibodies (2D1, 5HG and 2P7) were selected. All monoclonal antibodies were determined to be IgM using a EK-5050 kit (Hyclone).

In order to test that all monoclonal antibodies recognised IPGs, a non-competitive solid-phase enzyme immunoassay was used. F96 Polysorp Nunc-Immuno Plates are used for the assay. The polysorp surface is recommended for assays where certain antigens are immobilised.

The immobilised antigen (IPG) diluted to 1:800 captured the monoclonal antibody from tissue culture supernatant, ascitic fluid, and when the purified monoclonal antibody was used.

The detection method used an anti-mouse IgM, biotinylated whole antibody (from goat) and a streptavidin-biotinylated horseradish peroxidase complex (Amersham), ABTS and buffer for ABTS (Boehringer Mannheim).

The same immunoassay was used to evaluate the polyclonal antibody. In this assay, the detection method employed an anti-rabbit Ig, biotinylated species - specific whole antibody (from donkey).

The antibodies can be purified using the following

method. Fast Protein Liquid Chromatography (Pharmacia FPLC system) with a gradient programmer GP-250 Plus and high precision pump P-500 was used in order to purify a polyclonal IPG antibody.

5 A HiTrap protein A affinity column was used for purification of polyclonal IPG from rabbit serum. Protein quantitation was made using a Micro BCA protein assay reagent kit (Pierce).

Monoclonal IgM antibodies were purified in two steps.
10 Ammonium sulfate precipitation was the method chosen as a first step. Tissue culture supernatant was treated with ammonium sulfate (50% saturation). Pellet diluted in PBS was transferred to dialysis tubing before the second step.

Since ammonium sulfate precipitation is not suitable
15 for a single step purification, it was followed by gel filtration chromatography-antibody solution in PBS run into a Pharmacia Sepharose 4B column. Protein quantitation was made reading the absorbance at 220-280 nm in a Perkin-Elmer lambda 2 UV/VIS spectrophotometer.

20 Thus, this example shows that it is possible to raise monoclonal and polyclonal antisera to the A and P-type substances. These could be used as antagonists or binding agents.

25 **Discussion**

The material isolated by elution with 50 mM HCl (A-type IPG) inhibited cAMP dependent protein kinase and stimulated lipogenesis in rat adipocytes. This fraction also stimulated the proliferation of EGF-receptor
30 transfected 3T3 cells.

35 The biological characteristics of the A-type fraction isolated from human liver were recovered after treatment with pronase indicating that its activity is not due to either protein or peptides. The presence of phosphate and free amino groups suggests that these compounds could be similar to those reported to contain hexoses and hexosamines in their structure. The carbohydrate nature of

these compounds is supported by their behaviour in descending paper chromatography, characteristic of carbohydrate-containing compounds and resembling that of the IPG isolated from insulin stimulated rat tissues. All 5 experiments were consistent with the presence of A-type insulin-mimetic inositolphosphoglycans in the 50 mM fractions.

In humans, post-receptor tissue insulin resistance of glucose metabolism is a feature of non-insulin-dependent 10 diabetes mellitus (NIDDM) and many other disorders. Resistance could result from an intrinsic defect in insulin signalling pathways or could be caused by the presence of a circulating inhibitor of insulin action or both. Defects in IPG-associated mediator pathways therefore are key 15 targets for investigations on the pathogenesis of NIDDM.

The importance of IPG in insulin signalling comes from both *in vitro* and *in vivo* data. For example, mutant cells unable to make IPG respond to insulin by tyrosine phosphorylation, but without metabolic effects [1] and 20 cells bearing kinase-deficient insulin receptors do not hydrolyse GPI following insulin stimulation [2]. There is also a correlation with insulin receptor level with both insulin action and breakdown of GPI [3]. The insulin resistance of cells from diabetic GK rats, which have a 25 defect in GPI synthesis and release, can be overcome with IPG from bovine liver [4]. Similarly antibody to an enzymatically and chemically modified inositol phosphate glycan isolated from *Trypanosoma brucei* blocks the effects of insulin [5,6,7,8]. There is impairment of insulin-stimulated 30 hydrolysis of GPI in adipocytes from streptozotocin-diabetic rats and impaired insulin activation of pyruvate dehydrogenase (PDH) and glucose utilisation [5].

References:

The contents of all of the references listed below or mentioned in the description above are incorporated herein by reference.

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Appendix 1:1. IPG Hydrolysis

5 IPGs (LIA, LCA, LIP, LCP and blank), about the material obtained from 1 rat liver, was treated with 2N HCl (Pierce, extracted with dithizone as described below) @ 110°C for 90 min. After hydrolysis, the samples were freeze dried twice, redissolved in H₂O (200μl) and freeze dried once more. The material obtained was redissolved in 200μl 10 (H₂O) and 10μl samples were used to investigate the presence of inositols, monosaccharides and metals as described.

2. Dithizone Treatment of HCl

15 Diphenylthiocarbazone (Aldrich) was recrystallised from chloroform as described (Zief and Mitchel, p127). The crystalline purified dithizone was then dissolved in Cl₃CH @ 10mg/10ml and used to extract metal contamination from 6N HCl, constant boiling point (Pierce).

20 1ml of HCl was extracted with 500μl of dithizone solution three times and then used to hydrolyse IPGs as described above.

3. Placental IPGs. Extraction with Dithizone

25 50μl of stock IPGs solution were diluted to 200μl with H₂O and then extracted with 200μl of dithizone solution in chloroform (0.1g/l). (The chloroform used was extracted with H₂O/1N NaOH/H₂O just before preparing the dithizone solution.)

30 After extracting the IPG solution with dithizone (twice), the aqueous phase was extracted with Cl₃CH (200μl, three times). The organic phases were pooled and then washed with water, then dried and redissolved in Cl₃CH (200μl). The solution was extracted with 3N HCl (100μl) to determine metals.

35 The original aqueous IPG solution was used to determine changes in the Dionex Profile and PDH assay.

4. Dionex MethodsIPGs

Column and Guard Column: PA100
Eluants: A = 100mM NaOH
5 B = 500mM NaOAc + 100mM NaOH
Gradient: @ initial: 100% A, 0% B
@ 30 min: 25% A, 75% B
@ 30.1 min: 100% A maintained for 10 min
Flow rate: 1ml/min
10 Detector: ED40

5. Metal Analysis

Column and Guard Column: HPIC-CS5
Eluent: PDCA (6mm PCDA, 50mM AcOH,
15 50mM NaOAc, PH 4.57)
Flow rate: 1ml/min
Post Column reagent: 0.3mM PAR, 1M AcOH, 3M NH₄OH
Reagent flow rate: ~ 0.8ml/min
Detector AD40 @ 520nm

6. Inositol Analysis

Column and Guard Column = CarboPac MA1

Eluents: A = 500mM NaOH
B = H₂O

25 Conditions:

<u>Time</u>	<u>Flow</u>	<u>%A</u>	<u>%B</u>
Initial	0.25	25	75
0.00	0.25	25	75
15.10	0.25	25	75
30 20	0.40	100	0
25	0.40	100	0
34	0.40	25	0
35	0.40	25	75
40	0.40	25	75

35 Detector: ED40

7. Monosaccharide Analysis

Detector: ED40

Column and Guard Column = Carbo Pac PA1

Eluents: A=100mM NaOH; B=H₂O

5 Conditions: Flow rate: 1ml/min

	<u>Time</u>	<u>%A</u>	<u>%B</u>
	Initial	16	84
	0	16	84
	17	16	84
10	18	100	0
	23	100	0
	24	16	84
	30	16	84

15

20

Table 1:

Bioactivity of Mediators Per Tissue Weight

			<u>milliunits/g liver</u>
		PDH phosphatase (stimulating activity)	Lipogenesis (lipogenic activity)
5			
10	\$Human liver(10 mM Fraction)	1960 [N] 1650 [D]	no activity [N] no activity [D]
15	Human liver(50 mM Fraction)	600 [N] 700 [D]	2640±231 (n=3) [N] 551±119 (n=7) [D]
20	Human placenta (10 mM Fraction) Human placenta (50 mM Fraction)	28100 -----	----- no activity
25	†Rat liver (10 mM Fraction) (no insulin) Rat liver (10 mM Fraction) (plus insulin)	1992±157 (n=3) 3480±300 (n=4)	no activity no activity
30	Rat liver (50 mM Fraction) (no insulin) Rat liver (50 mM Fraction) (plus insulin)	1280 1090	1676±115 (n=2) 222±447 (n=3)
	¥Insulin (1 nM)		5160±310 (n=20)

Footnotes to Table 1.

t Unit of activity: A unit of activity is defined as the amount causing a 50% activation in the basal level of the test system.

† For the rat liver data the n value represents different independent extractions of separate liver preparations. Normally two animals (livers) were pooled prior to an extraction. Each lipogenesis assay was performed in triplicate. Two separate values are given, for control or sham injected rats or for livers extracted 2 minutes after an injection of 50 munits of insulin. Both groups were starved overnight.

¥ The insulin value is for twenty independent lipogenesis assays (each measured in triplicate) performed over the period October 1994 to October 1995.

§ The values for human liver are from two separate livers, [N] normal and [D] diseased. The diseased liver was obtained at the time of liver transplant. The normal liver was from a young healthy accident victim. The n values refer to repeat lipogenic assays. Separate extracts of the diseased liver which was kept frozen at -80°C were assayed over a one year period. No change in activity was found.

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Table 2:

Recovery of IPG from Affinity Supports

5	Control (cpm)	$209 \pm 79^*$
	10% FCS (cpm)	46313 ± 10231
	A-type (cpm)	33917 ± 6697
	P-type (cpm)	36542 ± 2278
10	C-18 (% recovery) †	
	A-type	86
	P-type	55#
	Blank (cpm)	$1377 \pm 317\text{f}$
15	AG3 (% recovery) †	
	A-type	11
	P-type	1.5
	Blank (cpm)	$505 \pm 61\text{f}$
20	AG50 (% recovery) †	
	A-type	1.5
	P-type	2
	Blank (cpm)	$258 \pm 114\text{f}$
25	Footnotes	

† ^3H -thymidine incorporation into the EGFR-transfected fibroblasts. A and P type mediators were eluted with water from the different supports. Final concentration of IPG was a 1/40 dilution of stock (see Materials and Methods). Similar results were obtained for dilutions of 1/80. All IPG stimulations were dose dependent.

Partial recovery of the P-type was consistently observed on C-18. No attempt was made to recover the bound material.

35 f Blank - Column eluate prior to elution of IPG.

* Control - Cultural medium only, no FCS.

Table 3
Negative MALDI Mass Spectroscopy Data

M/Z Table	MASS	FREQUENCY	Total Int:	138.278	Threshold: 20.41
		AMPLITUDE	REL.	INT.	RESOLUTION
	738.0299	96853.473	1.156	22.09	--
	751.0371	95175.928	1.125	21.50	--
	766.9858	93196.679	1.188	22.71	--
	773.0106	92470.236	1.746	33.37	--
	786.0075	90941.086	1.390	26.56	--
	794.9980	89912.555	1.601	30.60	--
	801.9772	89130.023	2.211	42.25	5600
	807.9960	89466.029	2.946	56.29	5600
	814.9775	87708.118	1.880	35.91	--
	820.9867	87066.082	2.586	49.41	5500
	949.0333	75317.765	1.640	31.33	--
	962.0265	74300.405	1.348	25.76	--
	964.9932	74071.960	1.196	22.86	--
	971.0243	73611.841	1.828	34.93	--
	972.0004	73537.912	1.219	23.30	--
	978.0016	73086.617	2.007	38.36	4300
	984.0213	72639.465	2.893	55.29	4600
	985.0138	72566.270	1.230	23.50	--
	993.9744	71912.014	1.429	27.30	--
	997.0110	71692.969	1.107	21.16	--
	999.9787	71480.175	3.165	60.47	4600
	1000.9930	71407.738	1.197	22.87	--
	1006.0019	71052.161	2.574	49.19	4500
	1006.9726	70983.661	1.995	38.12	--
	1012.9785	70562.755	3.923	74.97	4500
	1013.9822	70492.895	2.024	38.67	4100
	1017.9930	70215.129	1.105	21.11	--
	1018.9959	70146.016	5.233	100.00	4400

<<continued on next page>>

Negative MALDI Mass Spectroscopy Data

M/Z Table	MASS	FREQUENCY <<continued from previous page>>	AMPLITUDE	Total Int:	138.278	Threshold:	20.41
				REL.	INT.		RESOLUTION
	1019.9942	70077.352	2.211	42.	26	4600	
	1025.9652	69669.464	1.943	37.	12	4000	
	1026.9689	69601.369	1.243	23.	75	--	
	1027.9705	69533.539	1.466	28.	02	--	
	1028.9689	69466.067	1.235	23.	61	--	
	1031.9920	69262.552	3.157	60.	32	4400	
	1032.9977	69195.112	1.201	22.	95	--	
	1040.9588	68665.854	1.493	28.	52	--	
	1160.0469	61615.934	1.123	21.	46	--	
	1162.9966	61459.635	1.165	22.	27	--	
	1176.0016	60779.883	1.376	26.	29	--	
	1177.0109	60727.757	1.382	26.	41	--	
	1182.0249	60470.124	1.549	29.	59	--	
	1183.0144	60419.541	1.121	21.	43	--	
	1188.9999	60115.344	2.112	40.	36	3700	
	1190.0031	60064.661	1.303	24.	90	--	
	1191.0071	60014.016	1.098	20.	99	--	
	1195.0154	59812.695	2.251	43.	01	3900	
	1196.0143	59762.733	1.612	30.	80	--	
	1197.0000	59713.510	1.110	21.	36	--	
	1202.0095	59464.616	1.376	26.	29	--	
	1202.9869	59416.295	1.117	21.	34	--	
	1203.9951	59366.537	1.715	32.	76	--	
	1204.9813	59317.940	1.537	29.	37	--	
	1208.0132	59169.046	1.700	32.	48	--	
	1209.9891	59072.407	1.310	25.	04	--	
	1210.9788	59024.122	2.717	51.	92	3700	
	1211.9660	58976.037	1.529	29.	22	--	

<<continued on next page>>

Negative MALDI Mass Spectroscopy Data

M/I Table

MASS	FREQUENCY <<continued from previous page>>	Total Int:	138.278	Threshold: 20.41
		REL.	INT.	RESOLUTION
1215.9850	58781.087	1.195	22.84	--
1216.9945	58732.320	2.848	54.43	3700
1217.9747	58685.048	2.532	48.39	3800
1218.9427	58638.437	1.168	22.32	--
1223.9857	58296.808	3.593	68.65	3800
1224.9728	58349.744	2.076	39.66	3500
1225.9863	58301.499	1.118	21.37	--
1228.9816	58159.389	1.232	23.54	--
1229.9935	58111.534	4.112	78.57	3700
1230.9896	58064.506	2.816	53.80	3600
1236.9548	57784.448	1.398	26.72	--
1238.9621	57690.818	1.116	21.33	--
1242.9936	57503.679	2.623	50.13	3600
1243.9736	57458.373	1.317	25.17	--
1421.9781	50264.676	1.234	23.58	--
1423.0257	50227.665	1.108	21.17	--
1427.9938	50052.891	1.349	25.78	--
1428.9650	50018.869	1.202	22.97	--
1434.9910	49808.789	1.325	25.33	--
1436.0066	49773.557	1.094	20.90	--
1441.0083	49600.765	1.608	30.73	--
1442.0469	49565.037	1.107	21.14	--

Table 4
Negative MALDI Mass Spectroscopy Data

M/I Table	MASS	FREQUENCY	Total Int:	17.492	Threshold:	36.76
			AMPLITUDE	REL. INT.	RESOLUTION	
	738.0350	96853.582	1.145	42.42	--	
	751.0341	95177.094	1.047	38.79	--	
	766.9871	93197.298	1.051	38.94	--	
	773.0042	92471.788	1.147	42.51	--	
	788.9857	90598.558	1.144	42.37	--	
	795.0030	89912.776	1.418	52.54	--	
	801.9752	89131.030	1.752	64.93	--	
	807.9860	88467.911	2.699	100.00	5700	
	814.9730	87709.388	1.665	61.68	--	
	820.9815	87067.410	2.185	80.97	4900	
	1012.9684	70564.243	1.030	38.16	--	
	1018.9893	70147.259	1.208	44.77	--	

Table 5
Positive MALDI Mass Spectroscopy Data

M/Z Table	FREQUENCY	Total Int:	52.944	Threshold:	14.83
MASS		AMPLITUDE	REL. INT.	RESOLUTION	
844	.9287	84599.293	1.381	20.25	--
860	.9005	83029.630	3.100	45.43	5400
866	.9159	82453.450	4.840	70.94	5300
867	.9248	82357.589	1.532	22.46	--
1011	.9112	70637.738	1.182	17.33	--
1055	.9250	67693.060	1.812	26.56	4800
1058	.8859	67503.751	1.424	20.88	--
1064	.8906	67123.066	1.315	19.27	--
1065	.8751	67061.064	1.824	26.73	4200
1071	.8837	66685.104	4.090	59.95	4400
1072	.8817	66623.065	1.489	21.82	--
1077	.8985	66312.951	6.822	100.00	4200
1078	.9031	66251.196	2.852	41.80	4400
1266	.8959	56419.172	1.472	21.58	--
1269	.8440	56288.167	1.347	19.74	--
1275	.8661	56022.451	1.181	17.31	--
1276	.8342	55979.972	1.947	28.54	3600
1282	.8531	55717.288	3.084	45.20	3600
1288	.8797	55673.031	1.517	22.24	--
1289	.8763	55456.727	4.483	65.70	3500
1298	.8306	55031.794	1.092	31.23	3500
1499	.8771	47654.222	1.027	16.00	--
				15.06	--

Claims:

1. An isolated A-type substance as obtainable from human liver or placenta, wherein the substance is a cyclitol containing carbohydrate comprising Zn²⁺ ion and has the biological activity of regulating lipogenic activity and inhibiting cAMP dependent protein kinase.
5
2. An isolated A-type substance which is a cyclitol containing carbohydrate, said substance comprising Zn²⁺ ion, as obtainable by from human liver or placenta by:
 - (a) making an extract by heat and acid treatment of a liver homogenate, the homogenate being processed from tissue immediately frozen in liquid nitrogen;
 - 15 (b) after centrifugation and charcoal treatment, allowing the resulting solution to interact overnight with an AG1-X8 (formate form) anion exchange resin;
 - (c) collecting a fraction having A-type IPG activity obtained by eluting the column with 50 mM HCl;
 - 20 (d) neutralising to pH 4 (not to exceed pH 7.8) and lyophilising the fraction to isolate the substance;
 - (e) employing descending paper chromatography using 4/1/1 butanol/ethanol/water as solvent;
 - 25 (f) purification using high-voltage paper electrophoresis in pyridine/acetic acid/water; and,
 - (g) purification using Dionex anion exchange chromatography, or purification and isolation using Vydac HPLC chromatography to obtain the isolated A-type substance.
- 30 3. An isolated substance which is an A-type cyclitol containing carbohydrate comprising Zn²⁺ ion and has the biological activity of regulating lipogenic activity and inhibiting cAMP dependent protein kinase, wherein the substance has:
 - (a) a molecular weight determined using negative mode MALDI mass spectroscopy as shown in tables 3 and 4, or a
35

molecular weight related to one of the molecular weights set out in tables 3 and 4 by the addition or subtraction of one or more structure units of about 211m/z; or,

5 (b) a molecular weight determined using positive mode MALDI mass spectroscopy as shown in table 5, or a molecular weight related to one of the molecular weights set out in table 5 by the addition or subtraction of one or more structure units of about 211m/z.

10 4. The substance of any one of claims 1 to 3, wherein the substance comprises phosphate.

5. The substance of any one of the preceding claims, wherein the substance has one or more of the following properties:

15 (a) it migrates near the origin in descending paper chromatography using 4/1/1 butanol/ethanol/water as a solvent;

(b) it does not bind to a C-18 affinity resin;

20 (c) it is bound on Dowex AG50 (H⁺) cation exchange resin;

(d) it is bound on an AG3A anion exchange resin; or,

(e) the activity of the substance is resistant to pronase.

25 6. The substance of any one of the preceding claims, wherein the substance has the property of:

(a) inhibits adenylyl cyclase;

(b) mitogenic when added to EGF-transfected fibroblasts in serum free medium; or,

30 (c) stimulates lipogenesis in adipocytes.

7. A pharmaceutical composition comprising an A-type substance of any one of the preceding claims, in combination with a pharmaceutically acceptable carrier.

35

8. The pharmaceutical composition of claim 8, further comprising a P-type substance or insulin.

9. A substance of any one of claims 1 to 6 for use in a method of medical treatment.

5 10. An antagonist of the substance of any one of claims 1 to 6.

11. The antagonist of claim 10, wherein the antagonist has the property of:

10 (a) inhibiting release of the A-type substances;
(b) binding to A-type substances to reduce their level; and/or,
(c) reducing a biological activity of an A-type substance.

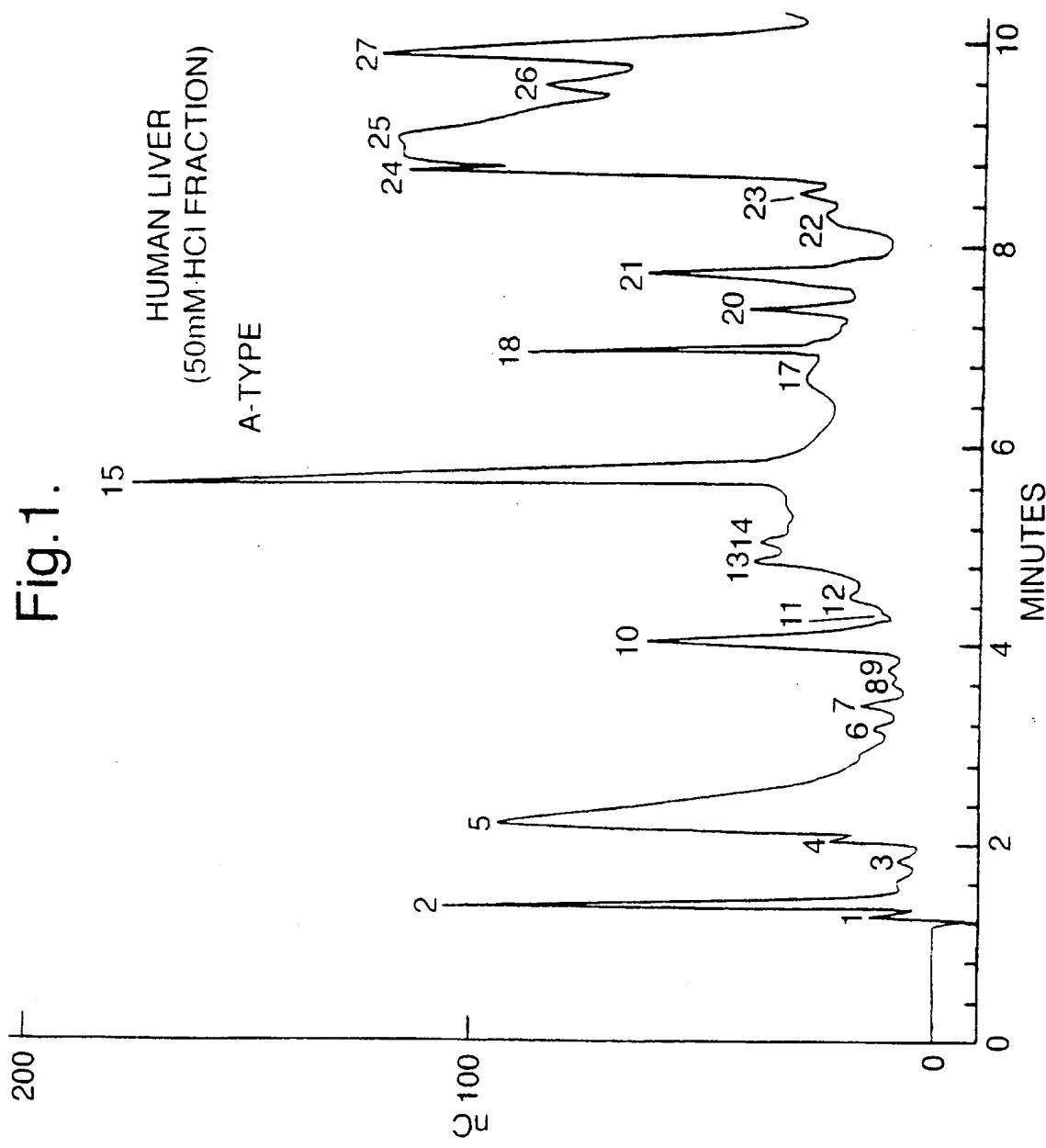
15 12. The antagonist of claim 10 or claim 11, wherein the antagonist is an antibody capable of specifically binding the substance or a binding protein capable of specifically binding the substance.

20 13. A pharmaceutical composition comprising an antagonist of any one of claims 10 to 12.

14. An antagonist of a substance of any one of claims 1 to 6 for use in a method of medical treatment.

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Fig. 1.



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Fig.2.

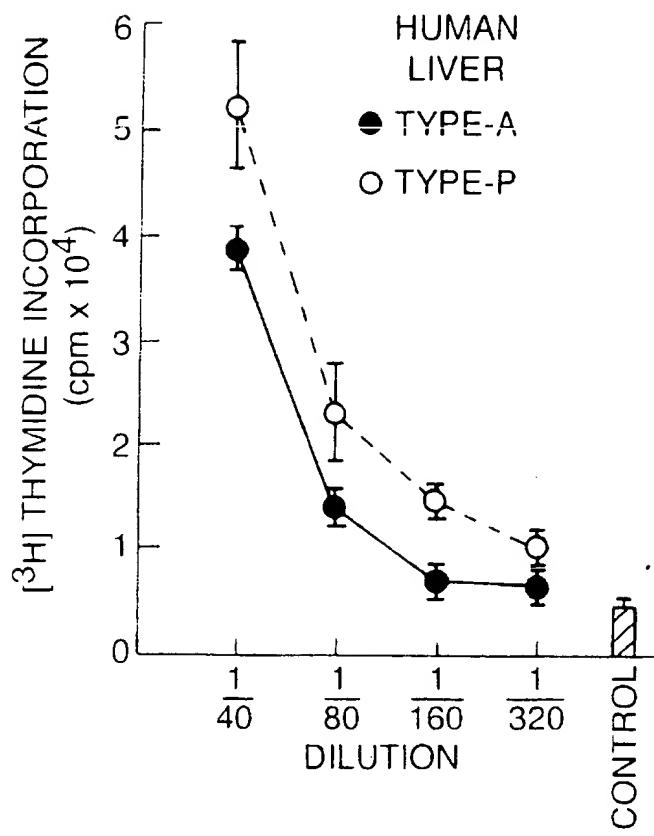


Fig.4.

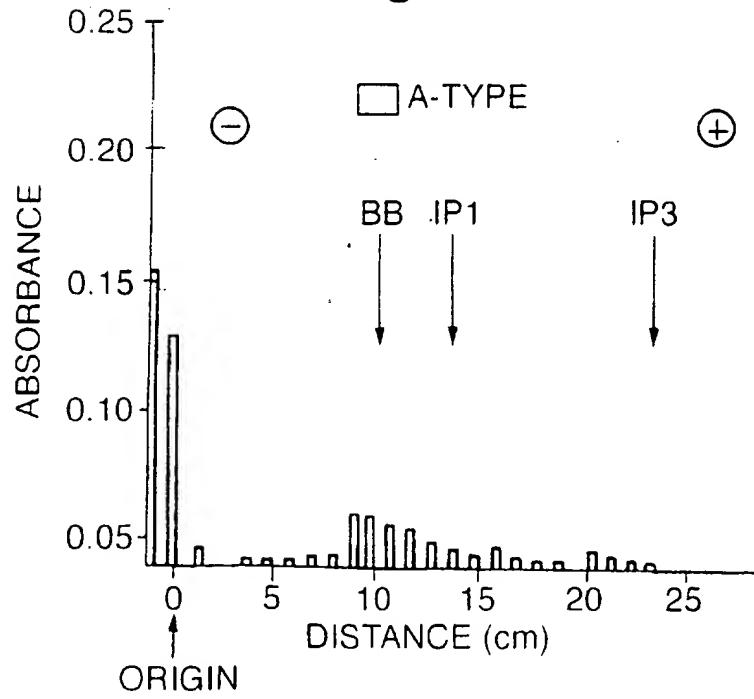
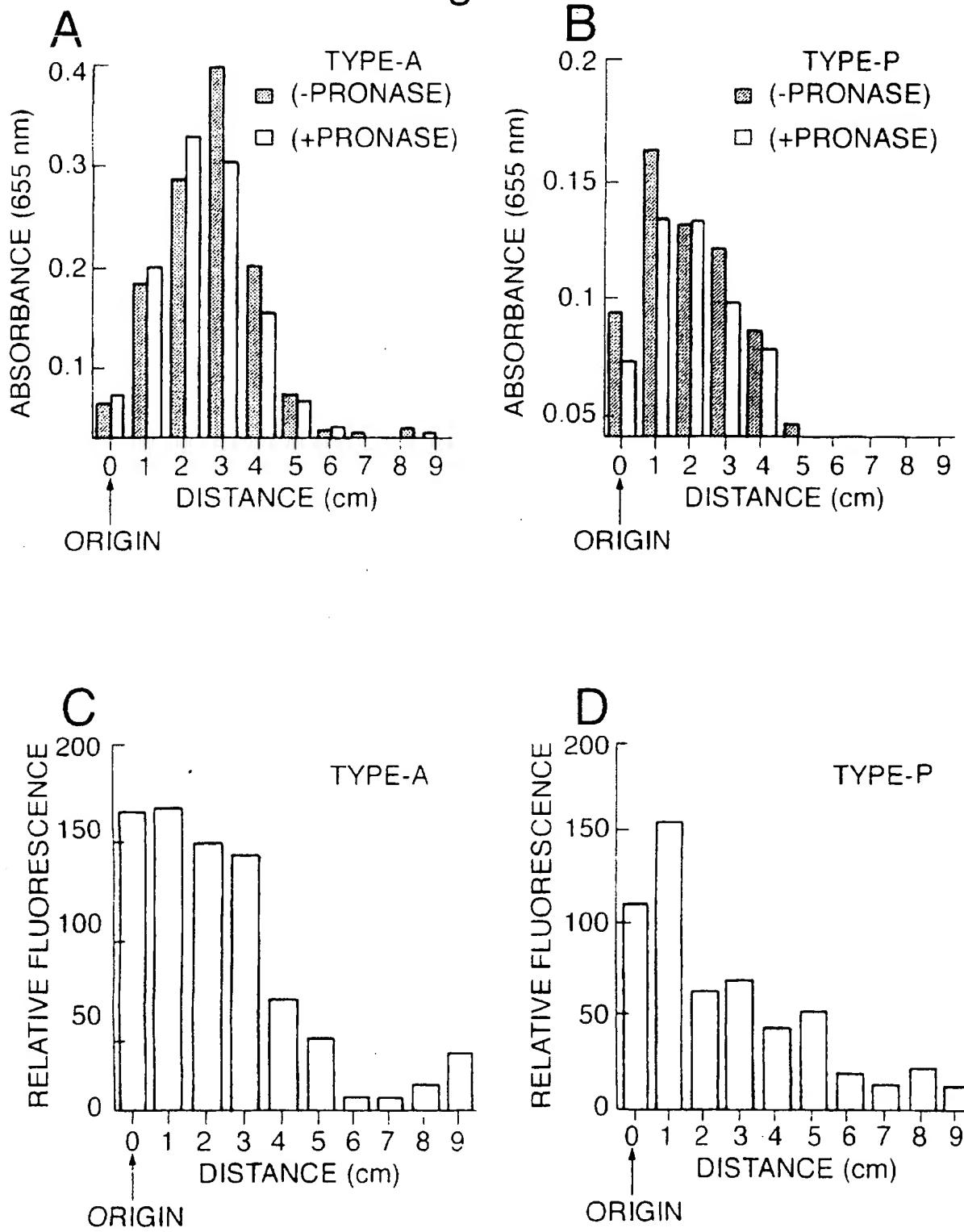
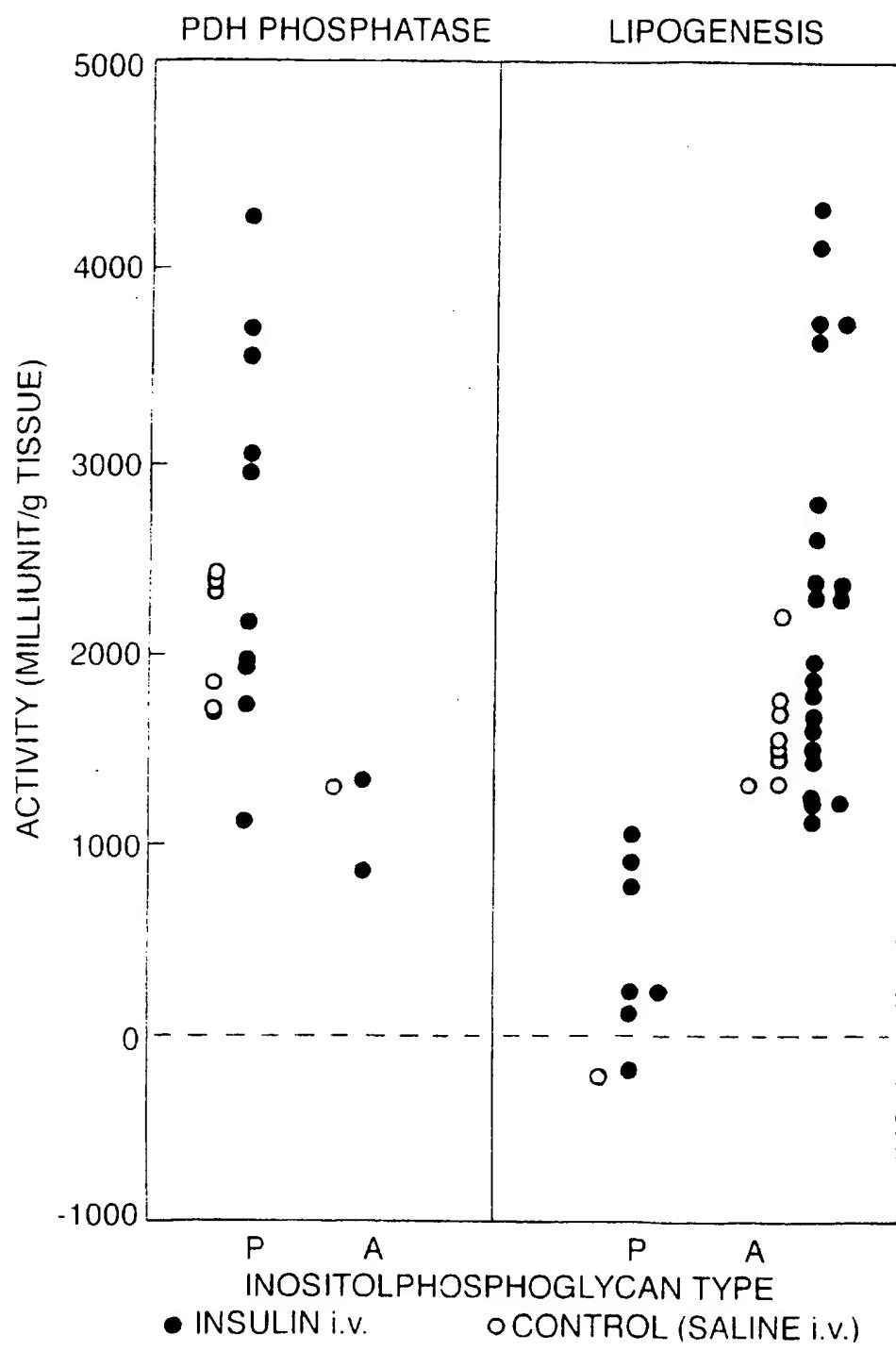


Fig.3.



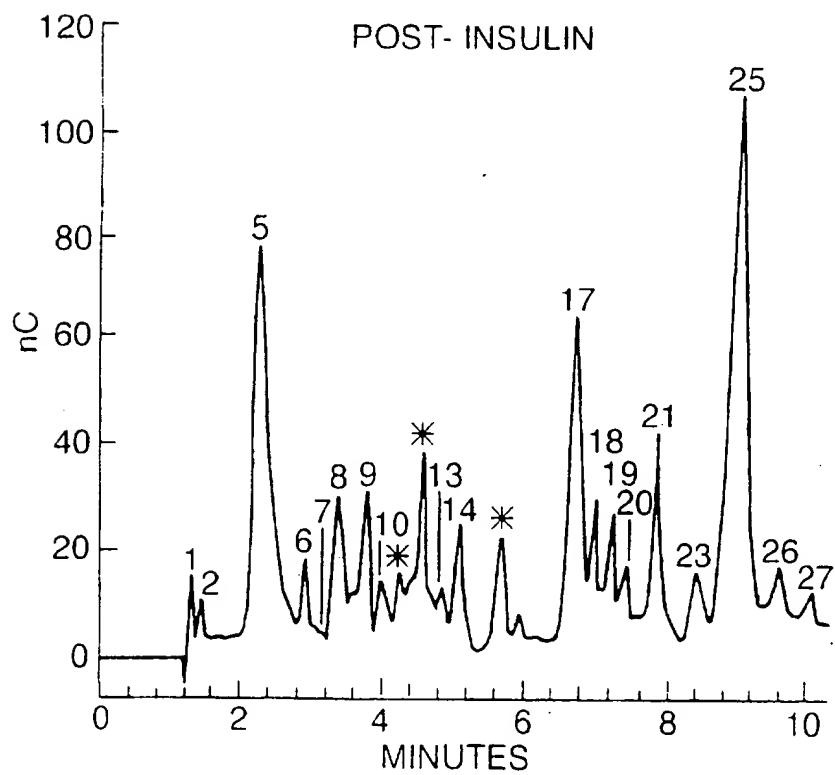
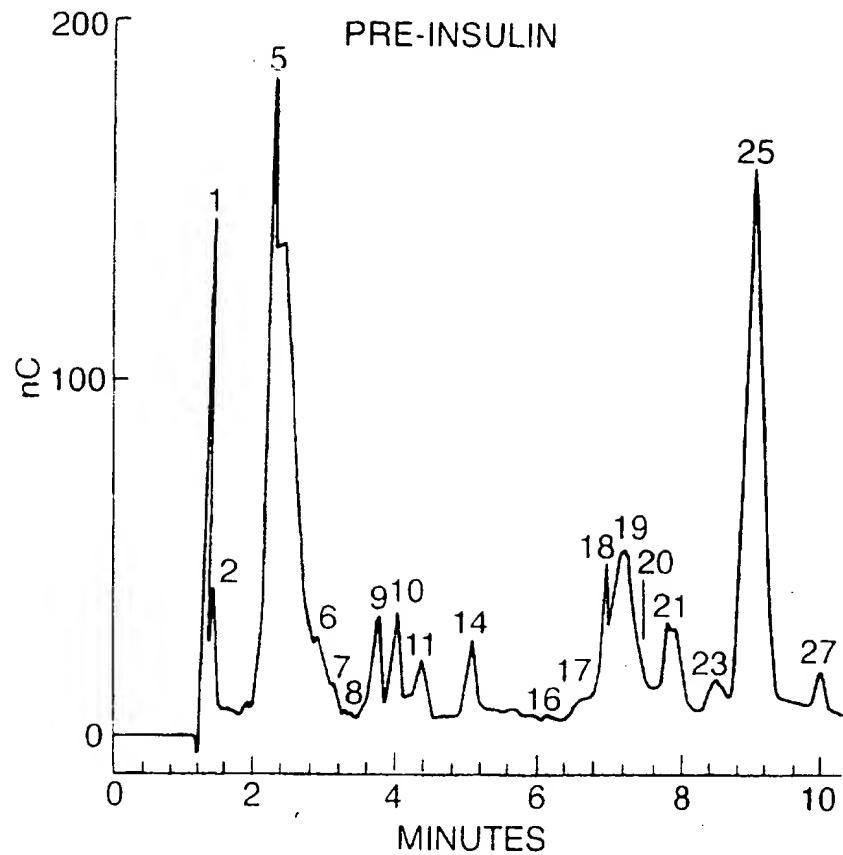
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Fig.5.



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Fig.6.



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Fig. 7.

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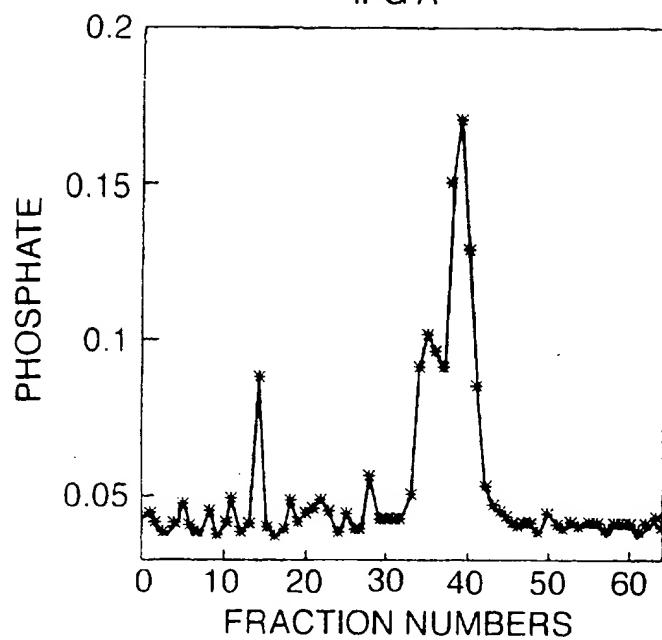
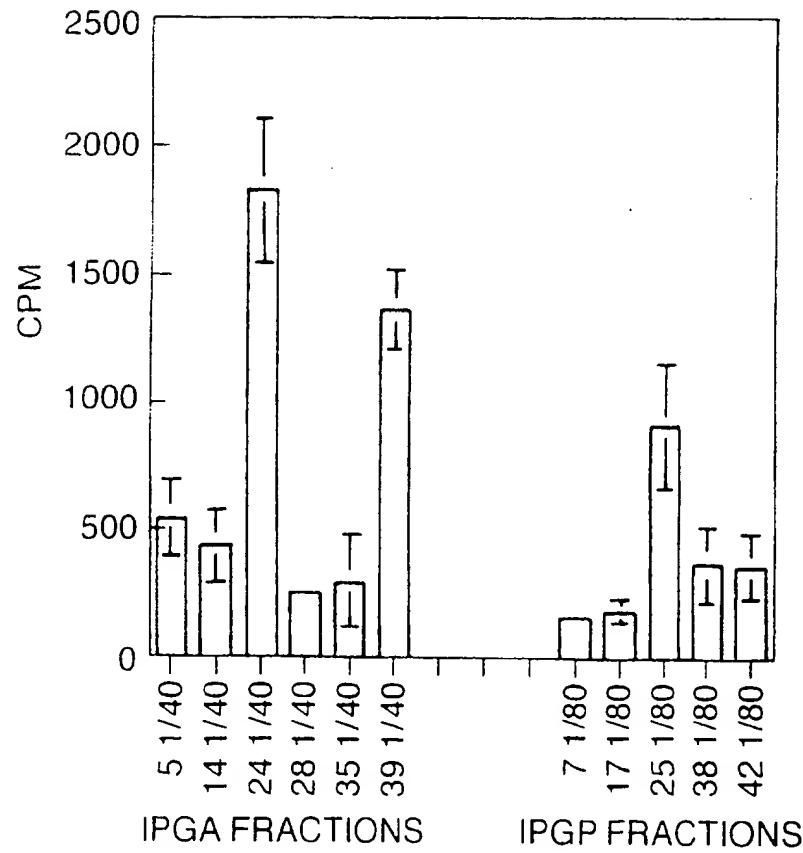
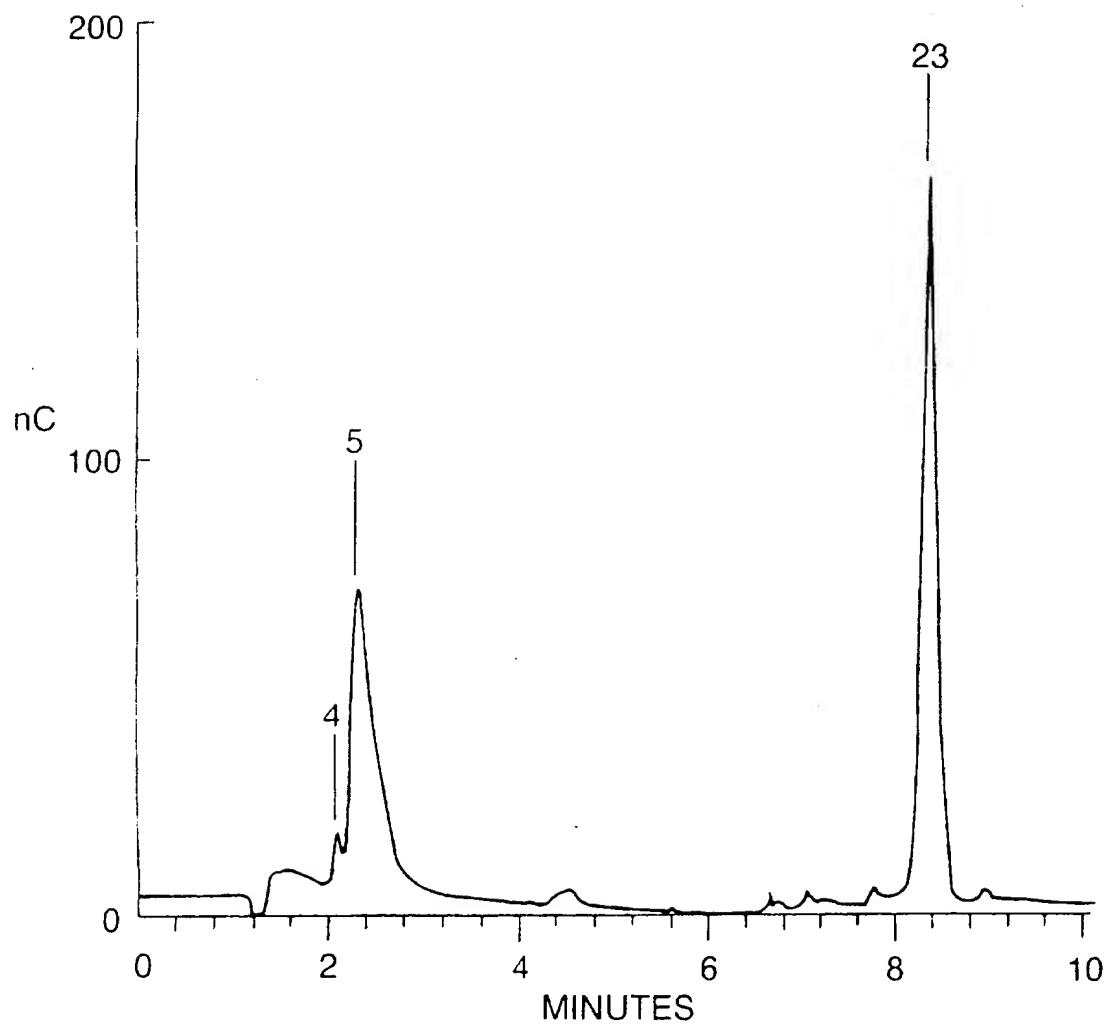


Fig. 8.



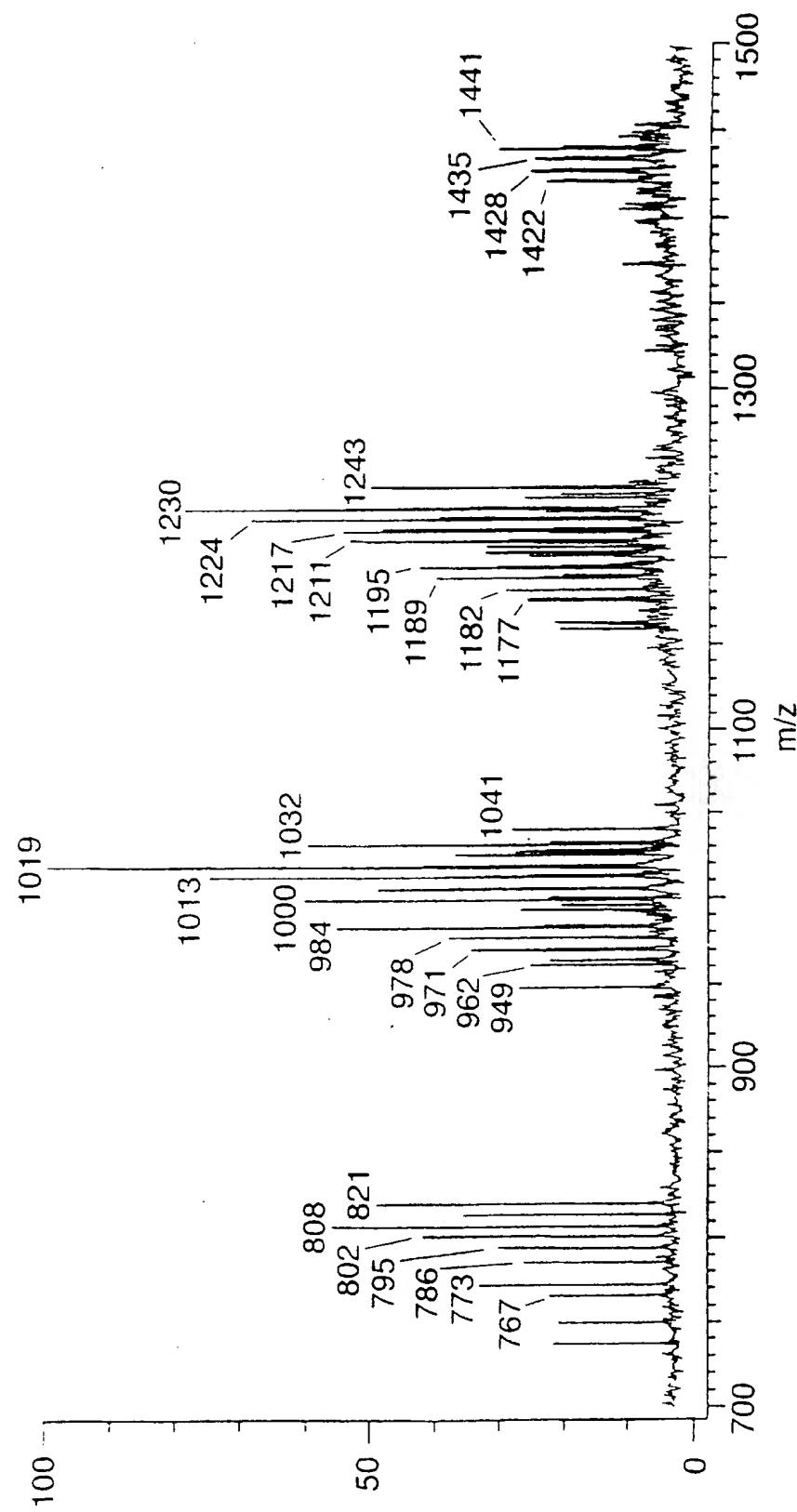
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Fig.9.



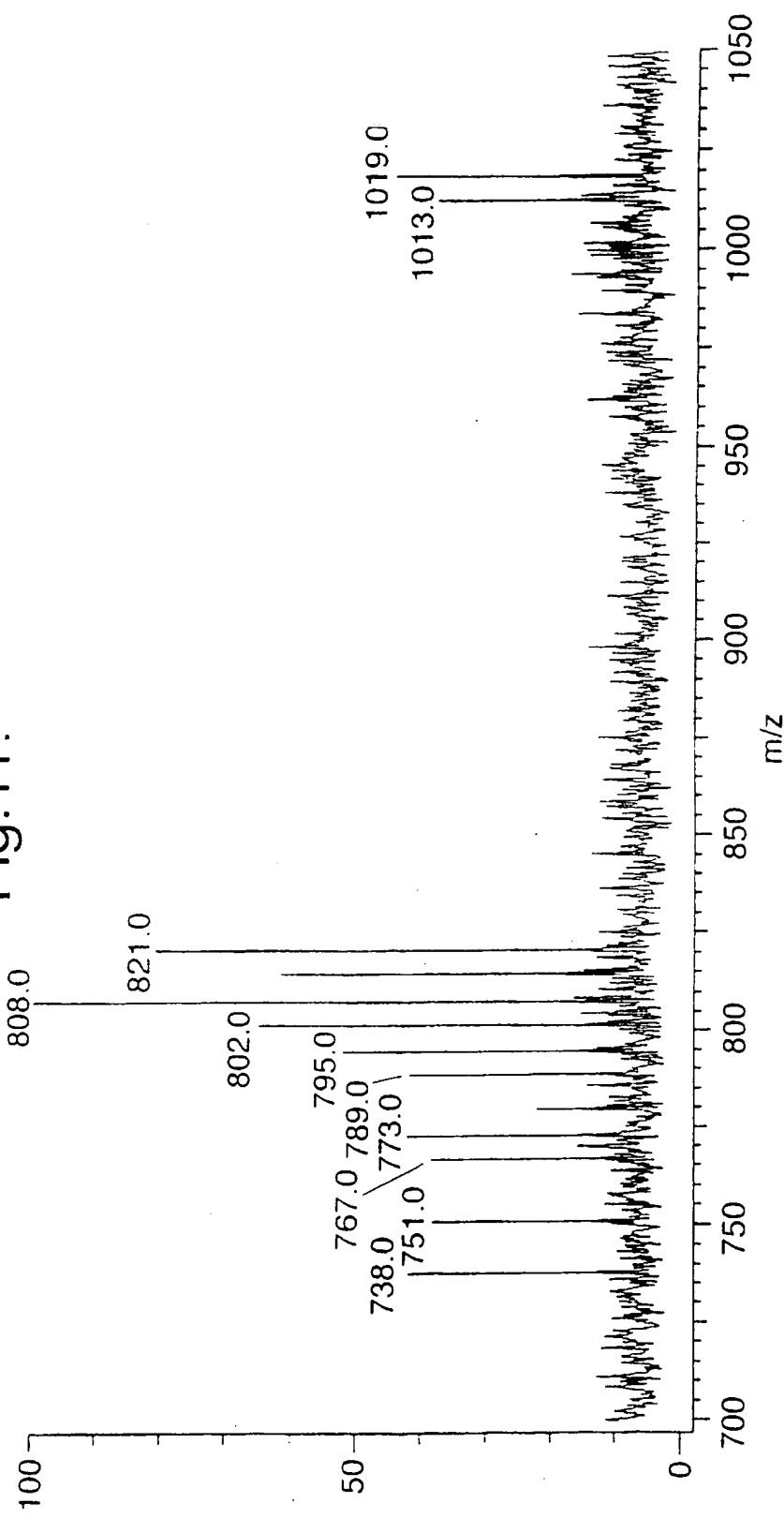
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Fig. 10.

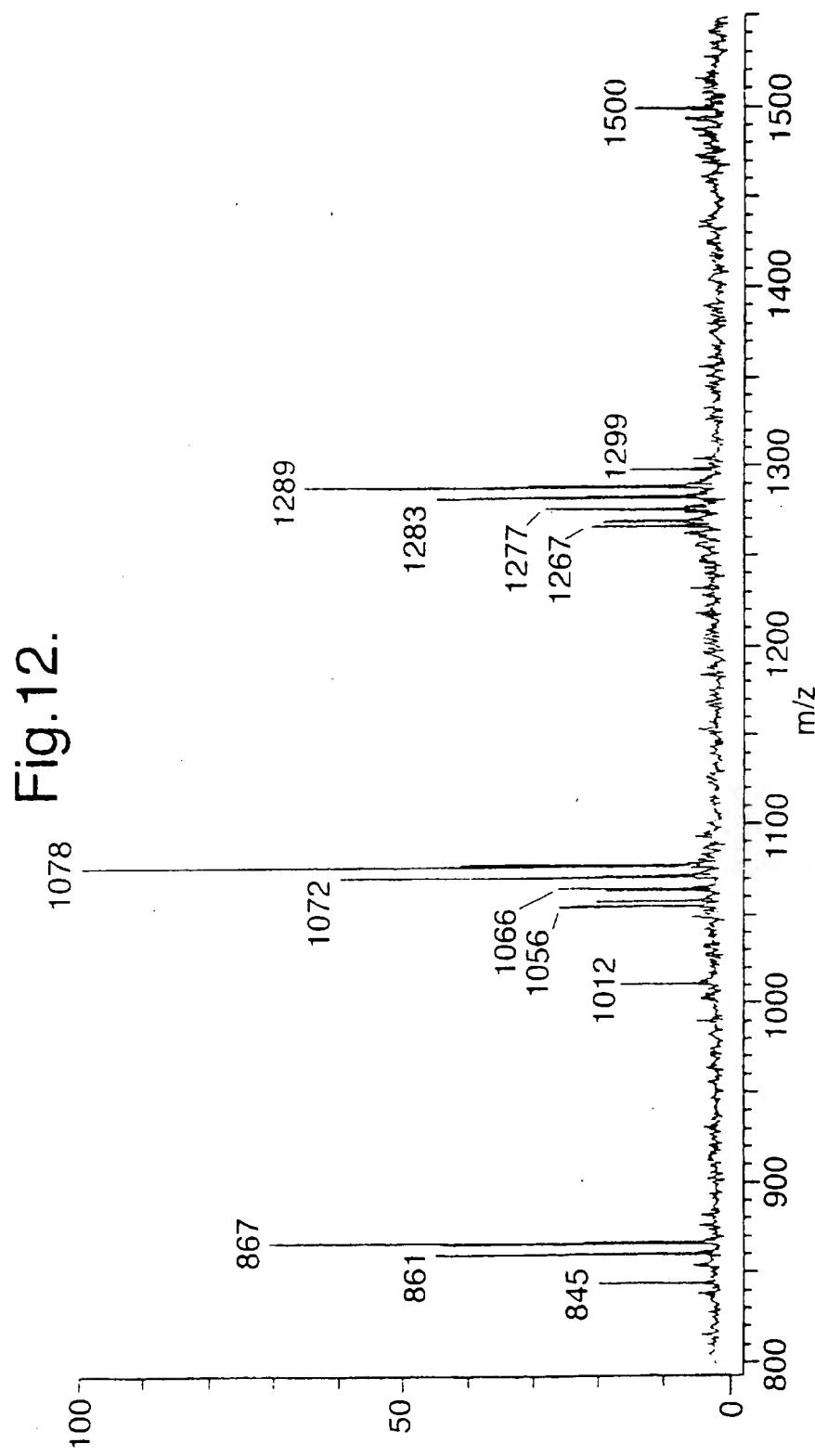


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Fig. 11.



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A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07G3/00 A61K31/70 C07K16/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07G A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	T. W. RADEMACHER ET AL: "Inositolphosphoglycan second messengers" BRAZILIAN JOURNAL OF MEDICAL AND BIOLOGICAL RESEARCH, vol. 27, no. 2, February 1994, pages 327-341, XP002050248 cited in the application see the whole document ---	1-3
P, X	WO 96 29425 A (UNIV LONDON ;RADEMACHER THOMAS WILLIAM (GB); CARO HUGO NORBERTO (G) 26 September 1996 see the whole document ---	1-3 -/-

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

' Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 December 1997	20/01/1998
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patenlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl. Fax (+31-70) 340-3016	Authorized officer Moreno, C

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 97/02444

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication where appropriate of the relevant passages	Relevant to claim No
P,X	H. N. CARO ET AL: "Isolation and partial characterisation of insulin-mimetic inositol phosphoglycans from human liver" BIOCHEMICAL AND MOLECULAR MEDICINE, vol. 61, no. 2, August 1997, pages 214-228, XP002050247 see the whole document ---	1-3
A	C. D. MALCHOFF ET AL: "A putative mediator of insulin action which inhibits adenylate cyclase and adenosine 3',5'-monophosphate-dependent protein kinase: partial purification from rat liver: site and kinetic mechanism of action" ENDOCRINOLOGY, vol. 120, no. 4, April 1987, pages 1327-1337, XP002050431 see the whole document ---	1-3
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A	EP 0 532 915 A (HOECHST AG) 24 March 1993 see the whole document -----	1-3

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